Factors effecting reproduction and larval development of Ostrea angasi (Sowerby 1871): Advancing hatchery production

By

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Declarations

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The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

Stephan Jon O'Connor

6th September 2015

Abstract

The flat or native oyster, *Ostrea angasi* (Sowerby, 1871*)*, was once a conspicuous inhabitant of many coastal bays and estuaries in south eastern Australia and formed the basis of a thriving wild fishery. Overfishing led to population collapses in NSW, Victoria, Tasmanian and South Australia, which in turn saw local demand for oysters being met through the production of Sydney rock oysters and the introduced Pacific oyster. As markets have developed and factors such as disease have impacted the production of other oyster species, there has been a renewed interest in production of *O. angasi* within the southern states.

O. angasi are sequential protandric hermaphrodites and larviparous, with brooding adults retaining larvae on the gill filaments in the brachial chamber for approximately half the larval life cycle. Wild catch of *O. angasi* spat is low and unreliable and the cultivation of this species is reliant upon hatchery production. To date production techniques for *O. angasi* have been loosely based on those used for *Saccostrea glomerata* and *Crassostrea gigas*, and have varied in success, with many hatcheries still reporting poor outcomes. In part this has arisen from a lack of systematic assessments of the fundamental variables that determine hatchery success. Specifically, improvements in *O. angasi* production output and reliability require an improved understanding of the reproductive biology of the species and knowledge of conditions required to optimise late larval development and metamorphosis.

In the first instance hatchery production is underpinned by the reproductive biology and ecology of this species. Knowledge of the temporal frequency of reproduction within New South Wales (NSW) estuaries is essential for larval acquisition and the development of a hatchery program. To address this, oysters were sampled over a 12 month period, across four estuaries, and examined histologically to reveal marked differences in gametogenic patterns to that previously reported in the southern states of Australia. The frequency of brooding oysters among farmed populations of *O. angasi*, in NSW waters, extended beyond that recorded in the Southern states of Australia. In the most northern and southern estuaries examined, brooding oysters were found between eight to nine months of the year with little synchronicity observed in reproductive timing within and between estuaries. Temperature,

previously thought to halt gametogenic activity, was not restrictive to *O. angasi* reproduction in NSW farmed populations.

For oviparous oyster species, assessments of larval rearing techniques frequently address specific ontogenetic stages, which begin with fertilisation success and culminate in competency and metamorphosis. For *O. angasi*, advanced stage larvae are acquired directly from brood-stock and greater emphasis is on the settlement and metamorphosis. This study sought to better understand the influence of the larval rearing environment on the morphological changes and physiological mechanisms associated with metamorphosis in *O. angasi*. Accordingly, techniques to reliably induce metamorphosis in competent larvae were assessed, and based upon these outcomes the impact of diet, temperature and salinity on later stage larvae were assessed, all with a view to improve *O. angasi* hatchery outcomes.

Several neuroactive catecholamines (epinephrine, epinephrine bitartrate, L-3,4-dihydroxyphenalalanine and gamma amino butyric acid) were trialed to induce metamorphosis of *O. angasi* larvae. The optimal dose and duration of exposure of epinephrine and epinephrine bitartrate was determined by induced metamorphosis of *O. angasi* larvae. The capability to induce rapid metamorphosis in competent *O. angasi*, aside from single seed production for industry, offers an invaluable tool to assess the effects of rearing conditions on larval development and acquisition of competency.

Using epinephrine induced metamorphosis, the influence of algal diet, rearing salinity and temperature on survival, growth and development of hatchery reared *O. angasi,* larvae was investigated. A comparison of dietary requirements for early stage larvae (140 - 230µm shell length) and late stage larvae (230 - 340µm shell length) was made. A series of uni, binary and ternary algal diets were assessed for their effect on the development of competency by induced larval metamorphosis. Optimal salinity (30 to 35) and temperature (26 to 29^{o}C) were determined for larval rearing, larvae reared outside these salinity and temperature range exhibited, reduced growth, survival and/or delayed development. The effects of short term (1h) reduction in larval rearing temperature from 26^{o}C significantly increased larval metamorphosis without affecting larval survival. However, short term increase in larval

rearing temperature from 26^{o}C adversely effected larval survival and metamorphosis, high lighting the need to assess rearing conditions during this critical stage of development. To ensure repeatability in outcomes, tests showed larvae sourced from different estuaries did not vary significantly in their metamorphic response to short term temperature manipulation and epinephrine induced metamorphosis.

Collectively the work in this thesis has significantly increased our understanding of *O. angasi* reproductive patterns and larval biology. The seasonal availability of larvae and the effects of the main environmental factors purported to influence reproduction of O. *angasi* have been examined. The environmental factors that can be easily and economically adjusted for greater larval rearing success were determined. Additionally, this work adds to the broader understanding of the acquisition of competency and larval metamorphosis in bivalve molluscs. Commercially, *O. angasi* hatchery outcomes have been highly variable; however, beyond having developed protocols for the use of settlement inducers, significant improvements in hatchery success have accrued. At the Port Stephens Fisheries Institute, a facility that has produced commercial quantities of *O. angasi* spat for over 15 years, the cumulative improvements in larval rearing technology described in this thesis have increased the percentage of larvae that successfully metamorphose from an historical average of approximately 57% for the four most successful rearing attempts to an overall average of 68% for nine larval rearing attempts.

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Statement of Co-Authorship

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Paper 1: **Located in *Chapter 3***: Chemically induced metamorphosis of hatchery reared flat oyster, *Ostrea angasi*, larvae by neuroactive catecholamines. 2009. Aquaculture Research, 40: 1567-1584.

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Chapter 1: General Introduction

1.1 Background

The native flat oyster *Ostrea angasi* (order *Ostreioda*, family *Ostreidae*, sub family *Ostreinae*), is a bivalve mollusc, previously classified as *Monoeciostrea sudaustralis* Orton, 1928 and *Ostrea rutipina* Tenison-Woods, 1878. It is commonly referred to as the flat, native, mud or Port Lincoln oyster and is considered physically and genetically similar to European oyster *Ostrea edulis* (Kenchington et al., 2002; Heasman et al., 2004). Members of the subfamily *Ostreinae* are stenohaline, inhabiting bays and coastal regions dominated by oceanic waters. *Ostrea angasi* is found attached to hard substrate or free on the sea floor, sub-tidally to approximately 40m (Edgar, 1997) and is distributed along the southern coast of Australia from Fremantle in Western Australia, Tasmania, through New South Wales (NSW) to Southern Queensland (Thomson, 1954) (Fig. 1.1). It has large triangular to round shells with unequal-shaped valves. Both valves can be thick, heavy, though friable, in older specimens. The inner surface of each valve is usually pearly white to light grey and smooth. The left valve is concave and right valve almost flat and has an undulating shell margin. The valve surface is rough and irregular with concentric growth lamellae giving the right valve a flaky, layered appearance. No teeth are reported however chomata may be present on the on the right valve in young shell and absent in older shell. The adductor muscle is approximately centrally located. Shells as large as 18cm across have been recorded (Boyd, 2011). Fossilised remains of *O. angasi* are known from late Plicocene calcarenites at the Roe Plain along the south-eastern coast of Western Australia (Morton et al., 2003).

Flat oyster species are commercially cultivated world-wide; *Tiostrea chilensis* in New Zealand, Chile and Europe; *Ostrea lurida* in the USA; and *O. edulis* in Europe. The largest flat oyster fishery is in Europe, where *O. edulis* is highly prized gastronomically and commands a high market price. Similarly, *T. chilenis* is highly prized in New Zealand where value of the "Bluff" oyster fishery is estimated to be over $20 million annually (Anon., 2010) and in Australia the farm gate prices for a dozen *O. angasi* averages $15.67 compared with $6.97 and $6.48 per dozen for Sydney rock oysters and Pacific oysters, respectively (Trenaman et al., 2015). Annual world flat oyster landings have declined in recent years from over 20,000 tonnes in the early 70s to approximately 2,500 tonnes in 2014 (FOA, 2013). Factors contributing to this decline are disease, overfishing and poor water quality. Both the European and New Zealand flat oyster industry declined in the late 70s and early 1980s due

to Bonamiosis caused by *Bonamia ostrea* (Pichot et al., 1979) and *Bonamia exitiosa* (Hickman and Jones, 1986) respectively.

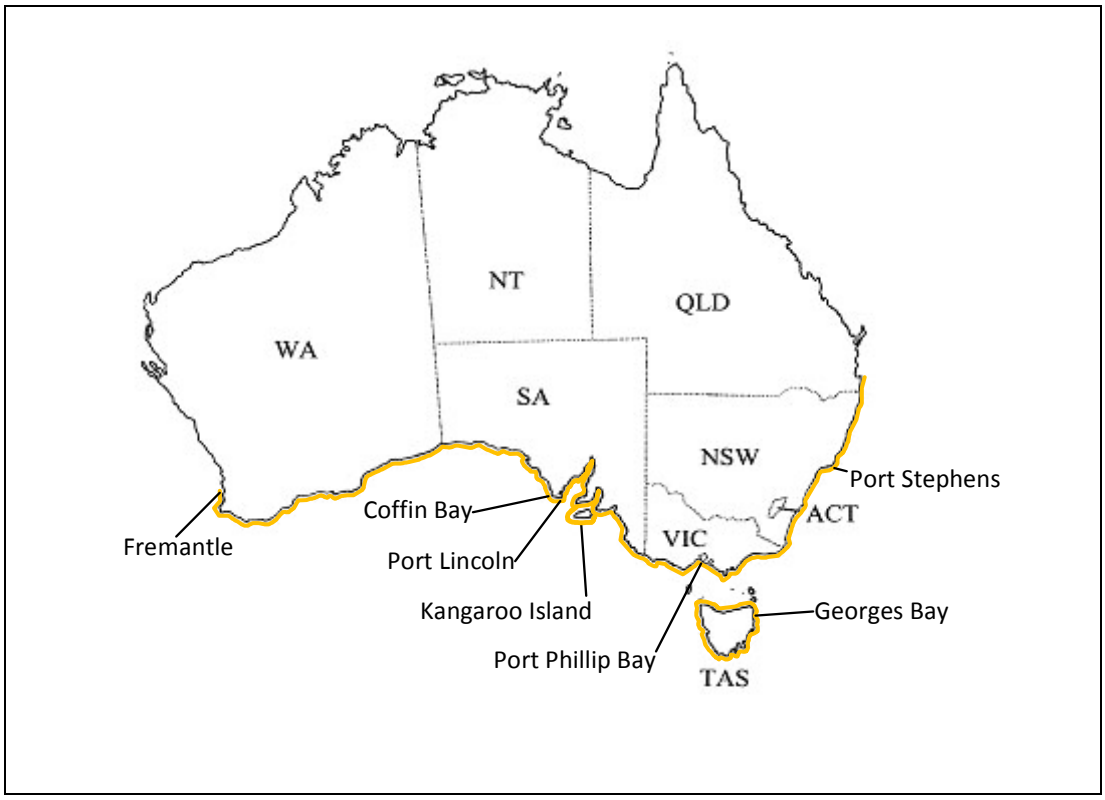

Figure 1.1 Geographical distribution of *Ostrea angasi* around the southern coast of Australia (yellow line) (Thomson, 1954).

The edible oyster industry in Australia began shortly after European colonisation and was based on harvesting wild *O. angasi* and Sydney rock oysters, (*Saccostrea glomerata).* Commercial exploitation of extensive, wild flat oyster beds began in the 1860s in Coffin Bay, Proper Bay, Vincent Gulf (Stansbury), and Kangaroo Island, South Australia (Olsen, 1994). Around the same time, inshore flat oyster fisheries were established in Tasmania (Sumner, 1972) and Victoria (Saville-Kent, 1891). The South Australian flat oyster fishery flourished for approximately 30 years before diminishing catches, due to over fishing, marked the

decline of the industry. In 1885 the Coffin Bay fishery was closed and by the early 1900s the remaining South Australian flat oyster fisheries barely existed (Olsen, 1994). An assessment of the Coffin Bay flat oyster beds in 1910 considered stocks had recovered sufficiently for the fishing to recommence (Randall, 1911); however annual landings continued to decline until the fishery ceased in 1948 (Grove-Jones, 1986). A survey of the Coffin Bay area by the South Australian Department of Fisheries in 1983 found the *O. angasi* populations had not recovered. A similar pattern of over exploitation and subsequent demise of the wild fishery occurred in Tasmania (Sumner, 1972). The only wild harvest fishery remaining for *O. angasi* is conducted in Georges Bay, North East Tasmania with 16,000 oyster hand harvested in 2012 (Anon, 2012).

The first recorded attempts to cultivate *O. angasi* were made in Proper bay, South Australia between 1910 and 1912 (Randal, 1911) and like later attempts by government and other private agencies, were unsuccessful (Thomson, 1952) due to the low and unreliable natural spat recruitment (Hodson, 1963). It was not until the mid 70s that hatchery production of *O. angasi* was attempted in Tasmania (Dix, 1976) and NSW (Anon., 1978). In the mid 80s, a pilot scale hatchery for *O. angasi* spat production was developed in Port Phillip Bay, Victoria (Hickman and O'Meley, 1986). Growth trials continued in Port Phillip Bay until 1991 when high mortalities from *Bonamia* spp. were recorded in the cultivated stock and trials ceased (Heasman and Lyall, 2000). Similar attempts to cultivate *O. angasi* in Western Australia were also halted by the occurrence of *Bonamia* spp. (Handlinger et al., 1999). Beginning in 1998 (Heasman and Lyall, 2002), the vast majority of recent *O. angasi* cultivation has occurred in NSW and has slowly grown to contribute approximately 4% of the State's total oyster production. Cultivation has also recommenced in South Australia in 2007, in Victoria since 2012, and sporadically in Tasmania. All production is hatchery based, however, production of this species in all states has challenges and an adequate, regular supply of spat at the optimum time for production has not been commonplace.

For at least a decade, increasing domestic and export market potential coupled with an oyster farming diversification strategy has driven the expansion *O. angasi* cultivation in Southern Australia. This expansion has been hatchery dependent and has highlighted fundamental gaps in our knowledge of the biology and ecology of *O. angasi*. From the outset, there has

been a paucity of information regarding the gametogenic patterns and spawning events of *O. angasi*, particularly in NSW, where the majority of production has occurred. This lack of knowledge has discouraged commercial hatchery production, limited spat outputs and has hampered the development of the industry. Hatcheries have been further discouraged from producing *O. angasi* spat by a comparative lack of well-defined larval rearing protocols, as this species has not been afforded the level of investigation given to other commercial oyster species. To date, rearing of *O. angasi* has been conducted using a patchwork of different larval diets and environmental conditions and this has undoubtedly limited hatchery success, particularly during settlement and metamorphosis. This is not atypical of *Ostreinae*, indeed the "Settle" project was established in 2009 to address these same problems with hatchery rearing of closely related flat oyster species *O. edulis* (Martinez-Castro, 2013).

There are two critical areas fundamental to hatchery production where current knowledge is inadequate. The first is the limited information about the spatial and temporal patterns of reproduction of *O. angasi*, in NSW in particular, which hampers the timing in collection of brood-stock for larval production. The second is the lack of systematic evaluation of factors affecting rates of larval development and metamorphosis for *O. angasi*. Together these knowledge gaps have limited the capacity of hatcheries to produce the quantities of *O. angasi* larvae required to support commercial production, while they focus on other commercial species with higher likelihoods of return.

1.2 Reproductive Biology

The reproductive biology for *O. angasi* is similar to that of other *Ostreinae*, being a sequential protandric hermaphrodite and larviparous (brooding). As brooders, *O. angasi* are fundamentally different to the other oviparous bivalves and present challenges in hatchery propagation of this species of larval collection for commercial production and the development of a selective breeding program (Jeffs, 1999; Joyce et al., 2013). Consequently, to plan hatchery operations knowledge of the reproductive ecology of a species is essential (Mann, 1979; Hickman and O'Meley, 1988a; Gonzalez-Arya et al., 2013, Joyce et al., 2013). The problems of unknown parentage and the potential for inbreeding has seen selective breeding program focus on pair mating to develop breeding lines. Current technology for

pair mating of any *Ostreinae* involves holding two oyster in aquaria for extended periods until larvae are released (Toro and Newkirk, 1990), a costly and time consuming process that has restricted breeding programs to small scale scientific attempts (Joyce et al., 2013). Additionally, the *in vitro* fertilisation techniques used for production of pair mated breeding lines with other oyster species have been largely unsuccessful with *O. angasi* and larvae must be collected from brooding adults.

1.2.1 Gametogenesis

The gametogenic phases of *O. angasi* are thought to closely resemble those described for *O. edulis* (Hickman and O'Meley, 1988a). Relatively little is known about the spatial and temporal patterns of gametogenesis and spawning activity of *O. angasi* compared to other commercially important bivalves. The histological examination of *O. angasi* conducted in Port Phillip Bay, Victoria reported temporal variation in gametogenesis; however the environmental conditions occurring during this survey were not reported (Hickman and O'Meley, 1988a). Because individuals can change sex several times in a season (O'Sullivan, 1980) and the sex ratios in the *Ostreinae* are often biased (Joyce et al., 2013) the selection for a male and female for a pair mated breeding line production is complicated. Though, nearly all one-year-old *O. angasi* individuals mature as males (S. O'Connor unpublished data); the selection of ripe female brood-stock is more difficult as the greatest proportion of ripe females recorded for *O. angasi* populations in Victorian waters was approximately 30% (Hickman and O'Meley, 1988a). Information regarding environmental factors that may influence temporal patterns of gametogenesis, sex determination and synchronicity of spawning events of *O. angasi* would assist in the development of the appropriate conditioning protocols for synchronised spawning for commercial hatchery production and the development of a selective breeding program. Further complicating brood-stock selection for *O. angasi* is the unknown proportion of hermaphrodites in the population and their contribution to the reproductive population has not been identified.

1.2.2 Spawning

In contrast to broadcast spawning characteristics of individuals in the genus *Crassostrea, O. angasi* females release oocytes that are forced up-current through the water tubules into the

inhalant chamber of the mantle cavity to rest loosely on the gill surface, where fertilisation occurs (Fig. 1.2). Following a brooding period, larvae are released into the water column for a pelagic phase of larval development (Dix, 1976; O'Sullivan, 1980). The duration of larval brooding and pelagic phase of flat oyster larvae is species dependent (Table 1). Ostried species also have less fecundity (6 thousand to 2 million larvae oyster^{-1}), compared to *Crassostrea* species (1 to >100 million eggs oyster^{-1}) (Mann et al., 2014). Fecundity of *O. angasi* is similar to *O. edulis* but greater than *T. chilensis* (Table 1.1). Female *O. angasi* release oocytes 130-150μm in diameter, similar to that of *O. edulis* but smaller than *T. chilensis* (280μm: Cranfield and Allen, 1977). The spermatozeugmata released by male *O. angasi* are negatively buoyant and is held in a radial array of sperm cells by an extracellur matrix to a core of acellular vesicles. The extracellular matrix is thought to inhibit sperm motility until the spermatozeugmata dissociates over a 24h period. The role of spermatozeugmata is thought to retain a greater localised concentration of viable sperm and also may be a stimulus for female spawning (Foighil, 1989).

Of the factors inducing spawning in bivalves, temperature is considered the most important (Sastry, 1968; Mann, 1979; Brown et al., 2010) followed by salinity, photoperiod, mechanical stimulation and water immersion time (Warwick et al., 1991; Dame, 1996; O'Connor et al., 2008; Paixao et al., 2013; Gomes et al., 2014). Spawning events for *O. angasi* are thought to be initiated when to water temperatures exceed 18^{o}C (O'Sullivan, 1980). Seasonal restriction of spawning events is not uncommon as in some bivalve species a spawning occurs when a threshold temperature has been surpassed or a definite temperature change occurs (Sastry, 1979). Anecdotally, *O. angasi* have been reported brooding in NSW outside the prescribed season for the southern states (Hickman and O'Meley, 1988a; Heasman and Lyall, 2000). This may allow larval collection in late winter or early spring and permit early season spat production. The production of spat early in the first growing season (early season production) can significantly reduce growing time and increase farm profitability (Hickman and O'Melly, 1988a). The possibility that the timing of gametogenesis of *O. angasi* individuals in NSW waters differs from the southern states warrants investigation as this may provide year round larval production.

1.2.3 Larval development

Rates of embryonic development, from fertilisation to "D" veliger, is dependent on environmental factors, mainly temperature and salinity (Bayne, 1965) and parentally derived endogenous energy reserves sequestered during gametogenesis to fuel the energetic demands of embryogenesis and early larval development (Helm et al., 1973). For *O. angasi* development from embryo to trochophore occurs within 24h at 23^{o}C and from embryo to the end of prodissoconch 1 developmental or "D" veliger stage, takes approximately 48hrs at 21^{o}C (O'Connor, unpublished data) to 96h at 15^{o}C (O'Sullivan, 1980). The stages of *O. angasi* larval development are thought to be similar to that of *O. edulis* larvae, where initial larval growth is lecithotrophic (to at least the "D" stage) and between "D" veliger stage and release, larvae are mixotrophic relying on both endogenous energy and planktonic derived particulate matter. *Ostrea angasi* brood between 1-2 million larvae per oyster for 15-18 days, after which time the larvae are released into the surrounding water column and are planktonic for approximately 10-15 days (O'Sullivan, 1980). The duration of both larval brooding and pelagic phases are dependent on environmental conditions, primarily water temperature, salinity, and food quality and quantity (Sastry, 1968).

1.3 Hatchery production

Knowing when and where sufficient larvae of the same stage of development can be collected from the brooding females is necessary for economical hatchery production of O. angasi spat. Following larval collection, identification of the most suitable larval rearing environment for hatchery production of this species is required. This includes determination of the most suitable larval rearing diet, optimal water temperature for growth and survival, and as most NSW hatcheries are located on estuaries experiencing intermittent freshwater input, optimal salinity for growth and survival. Assessment of larval rearing environment for growth and development is essential for each different species reared (Holland and Spencer, 1973; Devakie and Ali, 2000). A great deal of attention has been given to improvements in the hatchery production of other Ostreids, *O. edulis* (Mesias-Gansbiller et al., 2013) and *T. chilenis* (Jeffs, 1999) in the last decade, however little attention has been paid to the advancement of hatchery rearing techniques for *O. angasi*.

1.3.1 Brood-stock collection.

Ostrea angasi brood-stock are collected from the wild or farmed stocks only in late spring and early summer (October to December) and transported to the hatchery facility at the Port Stephens Fisheries Institute, Port Stephens. Oysters are opened manually and examined for the presence of larvae, which when found were rinsed from the gills and placed in tanks for cultivation. On rare occasions, brood-stock were placed in a recirculating system and fed in the hope that they would ultimately release larvae, this approach proved costly, unpredictable and inefficient, and was ultimately abandoned. In 2006, a more efficient method was developed that permitted larvae to be collected in the field. Magnesium chloride (50g L^{-1} in 25% sea water/75% fresh water: Butt et al., 2008) is used to narcotise brooding oysters and the larvae rinsed from the inhalant chamber without sacrificing the brood-stock. However, the constrained hatchery season (Oct-Dec), still limited, or on occasions, prevented spat supply. In contrast to *O. angasi*, a knowledge of the spatial and temporal reproductive patterns of Sydney rock oysters and their latitudinal variation throughout the State permits hatchery production from wild or farmed stocks 9-10 months of the year.

1.3.2 Larval rearing

Larvae that are collected from the parent during the mid to late stages of development, referred to as grey sick (Fig. 1.2) and black sick are generally selected for hatchery rearing to as rearing larvae from the early stages of cleavage, referred to as white sick (Fig. 1.2) is generally unsuccessful. Larvae are reared using procedures developed for the rearing of Sydney rock oyster larvae at the Port Stephens Fisheries Institute (O'Connor et al., 2008). Larvae are stocked in a 1,000L polypropylene tank at densities up to $5mL^{-1}$ and fed a mixed algal diet, the same as fed to Sydney rock larvae: *Isochrysis sp.* (T. Iso), *Pavlova lutheri*, *Chaetoceros muelleri* or *Chaetoceros calcitrans*. Larvae of disparate shell length cannot be cultured in the same rearing vessel as larvae are graded by sieving to get uniform shell length. Larval tanks are drained, cleaned and refilled with 1µm filtered seawater at 22-25^{o}C, every second day.

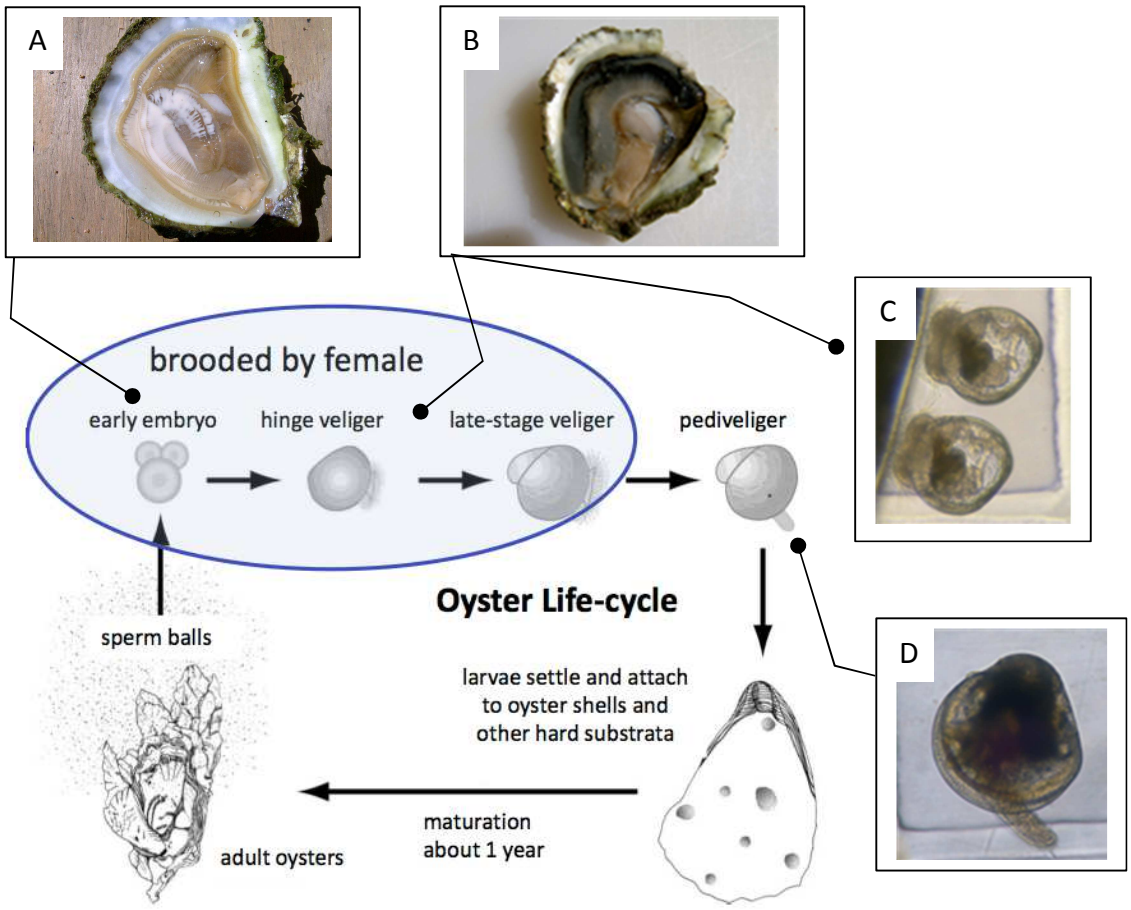

Figure 1.2 Reproductive cycle of *Ostrea angasi*. A. oyster brooding larvae in early stages of development ie white sick, B. oyster brooding larvae in early stages of development ie grey sick, C. grey sick larvae - straight hinged larvae and D. pedi-veliger. (diagram: South Slough National Estuarine Research Reserve, 2013. Photographs and micrographs: S. O'Connor)

Larvae are reared for 10-15 days to the "pediveliger" phase of development and are deemed competent and ready to metamorphose. Morphological structures used as indicators of competency of *O. angasi* larvae are primarily shell size (>340µm) and the development of eye spots. Behaviourally, larvae begin swimming with the foot extended and exhibit the classical swim/crawl exploratory behaviour of bivalve larvae. Competent *O. angasi* larvae

are placed in screens with ground, graded scallop shell (approx. 250µm particle size) in down-welling systems. The resulting spat are graded from the ground scallop shell when they reached approximately 400-500µm shell length. The spat are placed on 180µm nylon mesh screens and reared to approximately 1mm in shell length before transfer to estuary based up-welling units.

1.3.3 Larval competency and metamorphosis

Metamorphosis is a critical and energy consuming phase of early bivalve life history (Laing, 1995), particularly in hatchery reared larvae where substantial mortalities and variable levels of metamorphosis occur (Garcia-Esquivel et al., 2001; Pernet et al., 2006; Rico-villa et al., 2006; Perez-Parelle, 2013). For the hatchery production of any bivalve synchrony of the stage of larval development (i.e. pre or post competent) when treated with a metamorphic inducer is critical as decreased rates of metamorphosis have been reported if pre-competent larvae are exposed to metamorphic inducers (Hadfield, 1980; Avila et al., 1996). To avoid the potential of treating larvae to early assessment of larval rearing environment to maximise synchrony of larval development for the use of metamorphic induction is warranted.

1.3.4 Chemically induced metamorphosis

How larval competency is attained and the mechanisms by which metamorphic inducers work is poorly understood (Garcia-Lavandeira et al., 2005). Metamorphic cues appear to vary with mollusc species (Hadfield, 1978; Alfaro et al., 2011) and pharmacologically, different receptors types in involved in the metamorphic pathway have been identified in bivalve larvae (Coon and Bonar, 1987; Wang et al., 2006). However, not all competent bivalve larvae respond to metamorphosis inducing substances in the same way (Pawlik, 1990; Garcia-Lavandeira et al., 2005) necessitating the screening of different potential chemical metamorphic inducers and determining efficacy of dose and duration of exposure.
Traditionally, larval rearing concentrates on larval growth rates as the best indicator of optimal rearing conditions, however a better measure of larval rearing success is the successful transition through the critical stage of metamorphosis (Perez-Paralle, 2013). Water soluble chemical compounds can induce metamorphosis in a number of mollusc larvae, eg. *C. gigas* (Coon and Bonar, 1987), *O. edulis* (Shpigel et al., 1989; Garcia-

Lavandeira et al., 2005), *S. glomerata* (O'Connor et al., 2008), *Perna canaliculus* (Young, 2009) and clams (Garcia-Lavandeira et al., 2005). As chemical induction of metamorphosis is rapid, within 24h, and does not require larvae to adhere to a surface (Coon and Bonar, 1987), it enables the production of "single seed" spat, which are desired by industry. Though surface topography has been reported to induce high levels of metamorphosis in *P. canalicuclis* (Gribben et al., 2011), the use of chemical induction for metamorphosis of *O. angasi* is to specifically bypass surface attachment. Determining which substance that can chemically induced metamorphosis of oyster larvae can reduce production costs associated with hatchery rearing, provides single seed spat that allows easy quantification of production and reduce hatchery production times.

1.4 Thesis aims and outline

The overall aim of this thesis is to increase the knowledge base for the hatchery cultivation of *O. angasi*. The information arising from this thesis will not only increase the knowledge from the hatchery production of this species but assist in the understanding of the factors affecting the development of larval competency and metamorphosis. The knowledge gaps pertaining to *O. angasi's* reproductive ecology and larval rearing for hatchery production were addressed in four data chapters.

1.4.1 *Chapter 2*

Ideally, Hatcheries need to know when and where an adequate number of oysters brooding larvae of a similar stage of development are available for commercial production. The lack of knowledge regarding the temporal and spatial patterns of reproduction in NSW waters is problematic for larval acquisition in the field and for conditioning brood-stock in the hatchery. If hatcheries intend to undertake brood-stock conditioning or commence breeding programs to address threats such from *Bonamia* spp., then a greater understanding of the reproductive development of *O. angasi* is required. To address this paucity of information, farmed *O. angasi* were collected from four estuaries along a 700km latitudinal gradient of the NSW coastline over a 12 month period. Oysters were histologically examined to determine gametogenic status and presence and synchronicity of larval development recorded. As water temperature is considered the most important factor influencing reproduction in bivalves (Sastry, 1968; Mann, 1979; Brown et al., 2010) and fluctuations in salinity can induce

synchronised spawning of oysters (O'Connor et al., 2008), water temperature and salinity were recorded over the 12 month sampling period to determine if these environmental parameters influence *O. angasi's* reproductive ecology. This chapter examined the gametogenic cycle of *O. angasi* in NSW estuaries, and determined the timing for larval collection in the field and examined the influence of temperature and salinity on the gametogenic activity of *O. angasi* from four estuaries along a latitudinal gradient on the NSW coast.

1.4.2 *Chapter 3*

Larval rearing for the Ostried's has proved problematic around the world (Jeffs, 1999; Gonzalez Arya et al., 2012; Perez-Paralle, 2013) and problems still persist with hatchery production of *O. angasi* in Australia (S. Parkinson per comm., 2014; Xiaoxu Li, pers. comm., 2015). Larval settlement and metamorphosis in the hatchery can be protracted (Coon et al., 1990) and is a critical developmental phase when high levels of mortality have been recorded (Walne, 1974; Rodstrom, 1989; Hann, 1989; Rodriguez et al., 1990; Perez-Paralle, 2013). Transition through metamorphosis has become the measure by which larval rearing success is determined. A variety of chemicals are used to induce synchronous larval metamorphosis in mollusc hatcheries. Inducing rapid metamorphosis of competent larvae can reduce production costs associated with hatchery rearing by producing the desired number of spat and reducing time larvae and spat are retained in the hatchery. Chemical induction of metamorphosis is rapid and occurs without the larvae adhering to a substrate resulting in single seed (Coon and Bonar, 1987). Single seed allows early and easy quantification of spat produced in a hatchery, allows economical transport of spat to the farmers and the use of farming techniques that produce a marketable oyster sooner. This chapter examined the use of catecholamine and neuropeptides for the induced metamorphosis of competent *O. angasi* larvae and determines microscopically the larval morphological features that can be best used to determine competency in hatchery reared larvae.

1.4.3 *Chapter 4*

The importance of assessing the larval dietary requirements for hatchery rearing of different bivalve species cannot be understated (Aldana-Aranda and Patino Suarez, 1998; Rico-Villa et al., 2006) as considerable inter-specific variation occurs in bivalve larval dietary requirements (Coutteau and Sorgeloos 1992). Selection of algal species for bivalve diets has arisen from algal species availability, ease of algal culture, and success with culture of other bivalve larvae. Different diets have been used in attempts to rear *O. angasi* and on occasion, due to the incorrect selection of algal species, have not been successfully (G. Kent pers. comm., 2009). One of the more important aspects effecting the success of larval rearing, diet, has not been assessed for *O. angasi*. To address this gap in our knowledge of *O. angasi* larval rearing this chapter determined the effects of different algal diets on the growth and development of competency for hatchery reared *O. angasi* larvae using a metamorphic inducing substance

1.4.4 *Chapter 5*

Aside from larval diet, rearing temperature (Rico-Villa et al., 2009; Kheder et al., 2010) and salinity (His et al., 1989; Zimmerman and Pechenik, 1991; Dove and O'Connor, 2007) are important factors affecting larval survival and development. The measure of successful larval rearing is the quantity of larvae that survive metamorphosis to become juvenile spat. Methods to reliable induce metamorphosis of competent *O. angasi* larvae were developed and used to assess the effect of water temperature and salinity on growth, survival, synchrony of development to competency and metamorphosis. This chapter determined the effects of temperature or salinity on the growth and development of competency for hatchery reared *O. angasi* larvae using a metamorphic inducing substance.

Chapter 2: Reproductive ecology of the flat oyster, *Ostrea angasi* (Ostridea; Bivalvia) in four estuaries in NSW, Australia.

2.1 Introduction

The continued development of *O. angasi* farming is hatchery dependent due to limited and unreliable spat wild catch (Hickman and O'Meley, 1988a; Heasman and Lyall, 2000). *Ostrea angasi* is larviparous, necessitating the collection of larvae from a brooding female oyster for hatchery rearing. To accomplish larval collection reliably, either in the field or from hatchery conditioning systems, better knowledge of the association between seasonal changes of temperature, salinity and *O. angasi* condition indices and gametogenesis, fecundity and synchronicity of spawning is required.

Provision of spat in early to late spring can be advantageous for farming as this can increase the duration of initial growing season as long as suitable environmental conditions for growth are present. Early seasonal (spring) spat production can significantly reduce the time to harvest and increase farm profitability (Hickman and O'Meley, 1988a). Spring spat production is however, dependent of larval availability and obtaining larvae from the field is less costly than the more time consuming process of hatchery conditioning adults for larvae. In southern Australia, spawning and brooding of *O. angasi* is restricted to the warmer summer months and lack of larval availability necessitates hatchery conditioning for early spring spat production (Dix, 1976; Hickman and O'Meley, 1988a; O'Sulivan, 1980). Anecdotally, brooding oysters may be available in NSW for a longer period than in the southern states (Hickman and O'Meley, 1988a; Heasman and Lyall, 2000), and if correct, this may allow early spring spat production without the cost associated with hatchery conditioning. However, care must be taken in continually accessing hatchery produced farmed stocks for larval collection as parental contribution is unknown, the genetic diversity of the parental and larval stocks may be limited and the problems associated with inbreeding are likely (Naciri-Graven, 2000).

For hatcheries the synchronicity of spawning events is important as a greater frequency of brooding adults within the population at any given time increases the likelihood of obtaining

sufficient larvae at a similar stage of development for commercial production. Examining the reproductive activity of *O. angasi* populations experiencing different temporal patterns of temperatures and/or salinities may indicate which environmental variables can be manipulated in the hatchery to synchronise spawning events (Jeffs et al., 2002; Joyce et al., 2013; Gomes et al., 2014). Considerable information is available on reproduction for other Ostreinea (table 2.1), *Ostrea edulis* (Walne, 1974; Wilson and Simons, 1985), *Tiostrea chilensis* (Jeffs, 1998; Brown et al., 2010), *Ostrea nomades* (Siddiqui and Ahmed, 2002) and *Ostrea lurida* (Oates, 2013), but little is known regarding the reproduction of *O. angasi* and temporal availability of larvae in the northern extent of the species distribution.

Table 2.1 Fecundity and duration of larval phases for different oyster species from the subfamily *Ostreinae*, at different temperatures if information is available or provided in the publications.

Species	Temp °C	Fecundity x 10^5	Duration - days		Reference
			Brooding	Planktonic	
Ostrea lurida	24 18 -	2.5 - -	7 – 10 7 – 14 -	7 16 11 - 16	Loosanoff & Davis, 1963. Hopkins, 1937. Imai et al., 1954
Ostrea edulis	18 – 20 25 18 ± 2 21 ± 1	10 - - 0.14 – 0.78	6.5 – 10 - - -	7 - 8 8 - 12 12 – 14 -	Loosanoff & Davis, 1963 Laing, 1995 Mesias-Gansbiller et al., 2013 Millican & Helm, 1994
Ostrea puelchana	-	4.5 - 38	5 - 7	17 - 20	Pascual & Zampatti, 1995
***Tiostrea chilensis* (*O. lutaria*)**	-	0.06 – 1.03	21 - 30	2	Cranfield & Allen, 1977
Ostrea angasi	- 15 21	0.3 – 15.2 2.6 - 32 10 - 20	- 15 – 22 -	12 - 20 - 12 - 15	Dix, 1976 O'Sullivan, 1980 Hickman & O'Meley, 1988a

Aside from the benefits of early season spat production, hatcheries permit establishment of selective breeding programs (Nell, 2003; Culloty et al., 2004; Dove and O'Connor, 2011). Typical of *Ostreinae*, *O. angasi* is larviparous; eggs are released into the water tubules, forced up-current into the branchial chamber of the mantle cavity to rest loosely on the gill surface where fertilisation occurs (Morton et al., 1988). Approximately 1-2 million

developing larvae are retained on the gill filaments for 15-18 days (Hickman et al., 1986) before they are released into the surrounding water column. The larvae then have a feeding planktonic phase that lasts for approximately 15-22 days before settling and undergoing metamorphosis (O'Sullivan, 1980). The development of breeding programs for other *Ostreinae* has been complicated by their reproductive biology, specifically the inability use *in vitro* fertilisation techniques used in paired mated breeding programs, and difficulties in early larval rearing (Jeffs, 1999; Joyce et al., 2013). To overcome this problem, techniques for early larval rearing of *O. angasi* have been developed (O'Connor et al., 2012), but there has been little success with *in vitro* fertilisation. Additional to being larviparous, *O. angasi* is a protandric hermaphrodite and can change sex several times during a breeding season (O'Sullivan, 1980), further complicating the selection of individual oysters for pair mating. Obtaining males is not difficult as most *O. angasi* mature as males in their first year (S. O'Connor unpublished data), however information regarding environmental factors that can be economically manipulated that influence the frequency of females in the population would greatly assist a breeding program. Environmental factors effecting sex determination are little understood in the *Ostreinae* and the population structure in some species is predominantly functional hermaphrodites (Jeffs, 1998). However, if there is temporal variability in the presence of hermaphrodites in populations of *O. angasi*, this has not been reported (Hickman and O'Meley, 1988a) and their frequency within a population has not been assessed.

Given adequate food supply, temperature is considered the main factor effecting reproductive activity in bivalves (Sastry, 1968; Mann, 1979; Brown et al., 2010). On finer spatial scales, salinity, current flow, and immersion time affects reproductive activity in the estuarine environment (Warwick et al., 1919; Dame, 1996). Gametogenesis and spawning in *O. edulis* is temperature dependent, with the start and end of the breeding season strongly associated with water temperature (Joyce et al., 2013). Variation in reproductive activity along a latitudinal gradient has been proposed for most benthic invertebrates (Tranter, 1958; Lewis, 1986). The duration of breeding season increases with decreasing latitude for several commercial mollusc species, including scallops and pearl oysters (Heasman et al., 1998;

O'Connor, 2002). The latitudinal difference in reproductive patterns is thought to be temperature dependent, based on exposure to a critical minimum temperature that stops reproductive development, above which spawning can occur year round (Tranter, 1958). Evidence for this exists in other Ostreids, where Northern New Zealand populations of *Tiostrea chilensis* were reported to spawn and brood year round (Jeffs, 1998) in contrast to the defined seasonal peak in brooding oysters reported for more southern populations (Cranfield and Allen, 1977). Although the breeding season for *O. angasi* is during the summer months, only O'Sullivan (1980) indicated the requirement for a minimum water temperature of approximately 18^{o}C before spawning and brooding occurred. Little is known about the effects of water temperature or salinity on reproductive behaviour of *O. angasi.*

Oyster condition index (CI) has been used to compare oyster health between geographical separate shellfish populations (Lucas and Beninger, 1985; Mercado-Silva, 2005), to determine carrying capacity of aquaculture locations (Filgueira et al., 2014) and as predictors of reproduction and spawning in oysters (Walne, 1964; Joyce et al., 2013). For successful broodstock conditioning of *O. angasi* in the hatchery, individuals need to have good condition indices (wet weight condition indices >1000) or the process can be long and costly (Hickman and O'Meley, 1998b). Monitoring CI prior to spawning events will indicate if this physiological parameter can be used as a predictor for larval availability and the appropriate time to relocate brood-stock to the hatchery for conditioning for spring spat production or for selective breeding.

The aim of this work was to assess temporal predictability of larval availability in NSW and determine the environmental and physiological information that could be used to predict spawning events and may be of use in conditioning and synchronised spawning of *O. angasi* in a hatchery. This study described the temporal patterns of gametogenesis and brooding in populations of farmed *O. angasi* in four estuaries along a latitudinal gradient in NSW to assess differences in reproductive frequency and timing. In each location water temperature and salinity, CI, gametogenic phase, oyster fecundity and number of brooding oysters were monitored to examine the association between water temperature and salinity with CI and the

association between water temperature, salinity and CI with gametogenic phases, oyster fecundity and number of brooding oysters.

2.2 Materials and methods

Thirty hatchery produced *O. angasi* were randomly sampled monthly from farmed populations grown in four estuaries; Merimbula Lake, Merimbula (36^{o} 54' 07.72" S 149^{o} 52' 42.77" E), Bermagui River, Bermagui (36^{o} 25' 42.61" S 150^{o} 03' 18.80" E), and Wagonga Inlet, Narooma (36^{o} 12' 22.99" S 150^{o} 25' 53.90" E) all on the south coast of NSW, and Camden Haven River, Laurieton (31^{o} 38' 36.39" S 152^{o} 49' 54" E) on the mid north coast of NSW (Fig. 2.1). These estuaries provided a latitudinal gradient along the NSW coast and included the current southern and northern extent of *O. angasi* farming in NSW. All oysters sampled were a mix of different cohorts of spat that were hatchery produced at the PSFI in late 2003. Collections were carried out over 12 months in the first week of each month from July 2005 until June 2006. Mean monthly water temperature (temperature) and mean monthly salinity (salinity) data, from March 2005 to June 2006, were compiled from oyster farm records for the farm site within each estuary.

Oysters were transported live to the laboratory where biofouling was removed before shell length (DVM) and total weight of each oyster was recorded. The soft body tissue was removed from the shell and the soft tissue and shells were weighed. All weights were recorded to the nearest 0.01g. A wet weight condition index (CI) was calculated using the formula (Lawrence and Scott, 1982):

CI= (soft tissue weight (g) x 1000)/internal shell cavity capacity (g)

Where:

Internal shell cavity capacity = whole weight (g) – shell weight (g)

The branchial chamber of the oysters was inspected for the presence of larvae and when found, they were rinsed into a beaker with seawater and the total number was counted using a Sedgwick Rafter slide (x100 magnification). Larvae, which darken in colour as they develop, were classified according to the degree of development; "white-sick" for trochophores and newly developed D veligers (approx. 130-150µm DVM), "grey-sick" for first feeding larvae (approx. 150-190µm DVM) and "black-sick" for umbonate larvae (190-220µm DVM).

The proportion of oysters brooding larvae (brooding oysters) (P) for the reproductive period for each estuary was estimated using the formula (Cranfield and Allan, 1977, modified by O'Sullivan, 1980):

$$P=\sum_{i=2}^{n-1}\left(S_{t_i}\ (t_{i+1}-t_{i-1})/2\right)/\ddot{x}$$

Where S_{t_i} is the proportion of the population in the sample at time t_i that are brooding, n is the number of observations and $\ddot{x}$ is the brooding time. Two estimates of the brooding time were used for calculations, the first based on a maximum time for brooding of 22 days under natural conditions (after O'Sullivan, 1980) and the second based on a minimum brooding time of ten days under laboratory conditions (O'Connor et al., 2012).

For histologically examination of the gametogenic activity of farmed *O. angasi*, a 3mm transverse tissue section was excised adjacent and anterior to the adductor muscle. The tissue which was then fixed in Davidson's solution for 24h (Shaw and Battle, 1957) before being passed through graded alcohol solutions and then xylene. Tissues were embedded in paraffin, sectioned (5µm thick), mounted on acid washed glass slides, and stained with haemotoxylin and eosin (H&E). The gonad tissue was examined at x 200 magnification and the gametogentic phase of each individual was identified using descriptors provided by Cole (1942), Mann (1979), and Siddiqui and Ahmed (2002). The gametogenic phases were;

spent/spawning male and subcategories: developing male, ripe male (Fig 2.2 A, B & C respectively), spent/spawning female and subcategories: developing female, ripe female (Fig 2.3 A, B & C respectively), predominantly male (most of the gametes present were sperm) hermaphrodite, predominantly female hermaphrodite, (most of the gametes present were oocytes) and non-defined (note: oysters considered to have residual male gametes were included in non-defined) (Figure 2.4 A, B &C respectively).

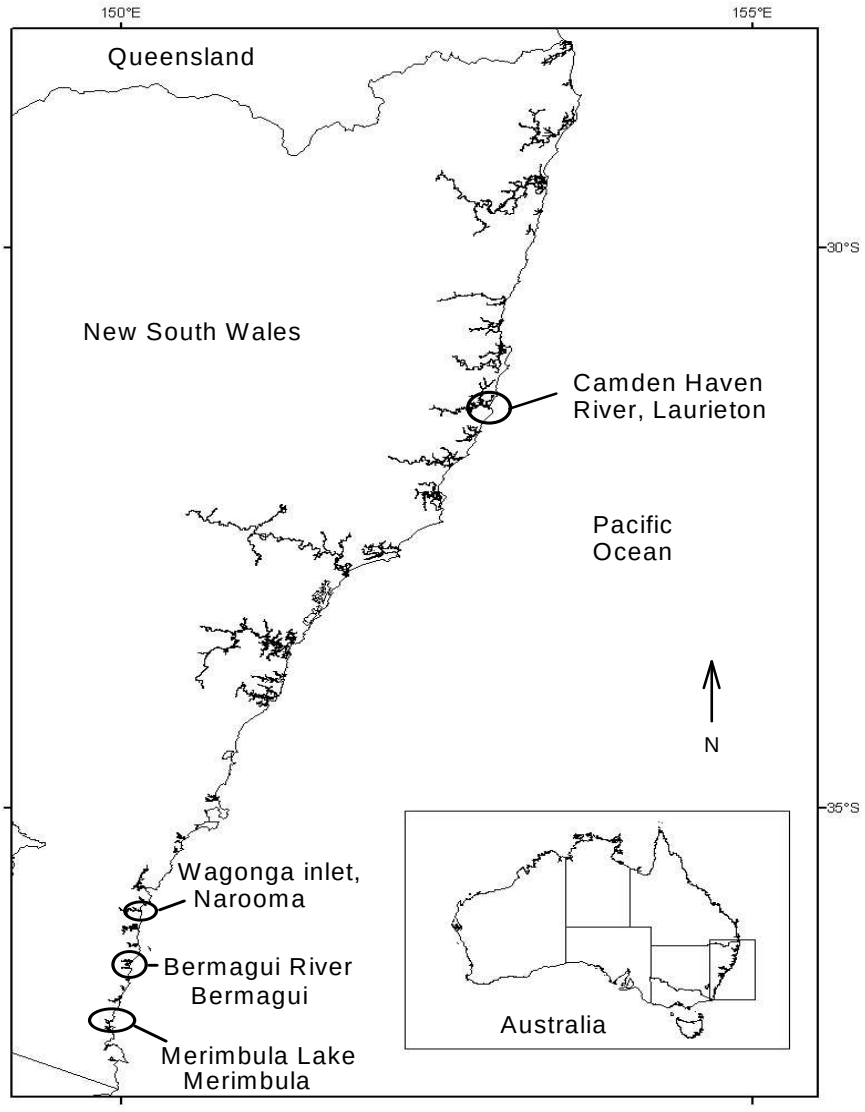

Figure 2.1 Location of estuaries along the New South Wales coast with *Ostrea angasi* farms from which monthly collections were undertaken.

2.2.1 Statistical analysis

All statistical analysis was conducted using SPSS v.22.0 software (SPSS Inc. 2013, Chicago Illinios, USA). For analysis of variance (ANOVA) when necessary, percentage data was arc sin square root transformed or log transformed to correct for heterogeneity of variance. Homogeneity of variance was checked using Cochran's test and residual plots. Where significant treatment effects were found, pair-wise comparisons among means were made using Tukeys HSD (Sokal and Rolhf, 1981). Acknowledging the influence of immediate past environmental history on oyster physiological variables, correlations in each case were assessed with the records from the previous month and/or the month before that (-2 month). For statistical analysis involving temperature, salinity or CI, as oysters were sampled on the commencement of each month, the temperature, salinity or CI from the previous month and from -2 month were used. Monthly means were used from the same month and previous month for statistical analysis involving brooding oysters and the gametogenic phases of spent, non-defined and spent & non-defined. All mean values are ± standard error (SE) and in all analysis $\alpha = 0.05$.

2.2.2 Within estuary comparison

One way ANOVA was used for each estuary to determine whether the average CI and the average number of larvae per brooding oyster differed among months. A Chi-squared test of independence or Fischer exact test, where appropriate, was used to assess differences in the frequency of brooding oysters and histologically determined phases of gametogenesis from each month of male (combined developing and ripe male), female (combined developing and ripe female), hermaphrodite (hem), spent and non-defined oysters from each estuary for each month of the 12 months sampled (standardised residuals used to determine departure from expected values). Pearson product-moment correlation coefficients were used to investigate potential relationships between the physiological observations (CI, frequency of gametogenic phase and brooding oysters) and the environmental variables recorded (temperature and salinity). Specifically, changes in:

1. condition index and temperature or salinity from previous & -2 months.

2. condition index, temperature or salinity from previous & -2 months and the frequency of the major gametogenic phases of male, female, spent, non-defined, spent & non-defined, and hermaphrodite oysters.
3. the frequency of brooding oysters and CI, temperature or salinity from previous & -2 months.
4. the frequency of the gametogenic phases of spent, non-defined or spent & non-defined oysters combined and the frequency of brooding oysters from same & previous months.
5. the frequency of the gametogenic phases of developing & ripe males, developing & ripe females, spent, non-defined, spent & non-defined or brooding oysters and the frequency of sub category of hermaphrodites - hermaphrodite male, hermaphrodite female or hermaphrodite equal oysters from previous month and following month.

2.2.3 Among estuary comparison

One way ANOVA was used assess if CI or the average number of larvae produced per brooding oyster among each estuary varied for the 12 months sampled. A Chi-squared test of independence or Fischer exact test where appropriate, was used to assess differences in the frequency of brooding oysters; the ratio of the gametogenic phases of brooding oysters; the ratio of gametogenic phases of male, female, hermaphrodite, spent, non-defined oysters; differences in the ratio of gametogenic phases of developing and ripe male and female oysters from each estuary for the 12 months sampled combined (standardised residuals used to determine departure from expected values). No comparative analysis was made among estuaries by months sampled due to the confounding factor of farming activity. The farming practices between estuaries, including frequency grading, different stocking densities and "rack height" (height of cultivation equipment in relation to mean low tide) all differed, however *O. angasi* stock sampled were graded and farmed at the same frequency, density and rack height within estuary.

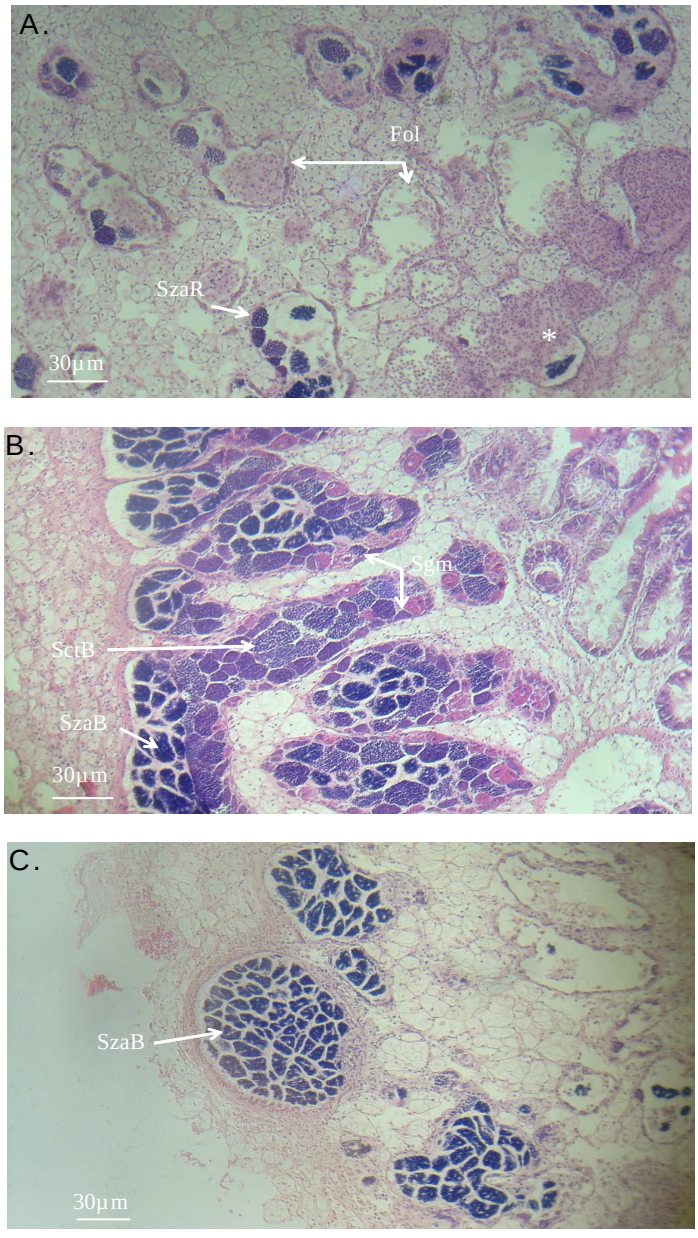

Figure 2.2 Micrographs of histological sections of *Ostrea angasi* gonad showing **A**. Spent/spawning male: Follicle (Fol) containing residual spermatozoa (SzaR) and haemocytes surrounded by connective tissue (*). **B**. Developing male: spermatagonium (Sgm), spermatocyte balls (SctB) and few spermatozoa balls (SzaB) present. **C**. Ripe Male: spermatazoa balls filling follicle, few spermatocyte balls maybe present but no spermatogonium present.

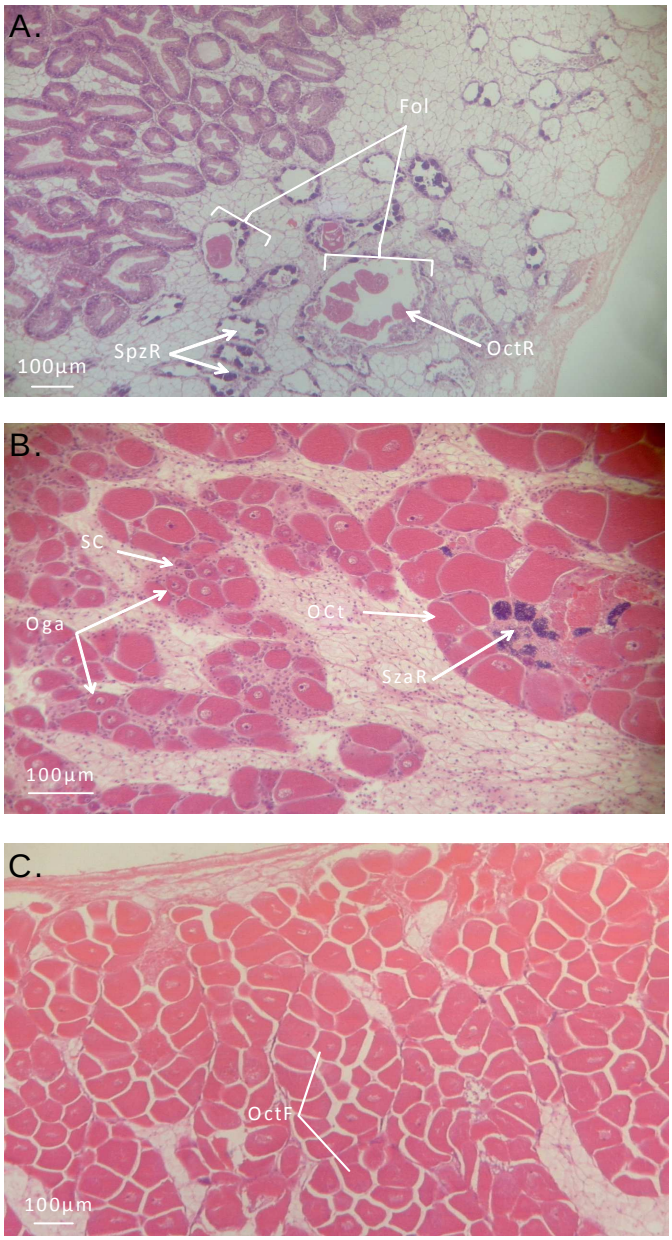

Figure 2.3 Micrographs of histological sections of *Ostrea angasi* gonad showing **A**. Spent/ spawning female: Follicle (Fol) containing residual oocytes (OctR) and spermatozoa (SzaR). **B**. Developing female: oogonia (Oga) arising from stem cells and numerous developing oocytes (OctD), no free oocyte with residual spermatozoa (SzaR) present. **C**. Ripe female: free oocyte (OctF) filling the lumen of the gonad.

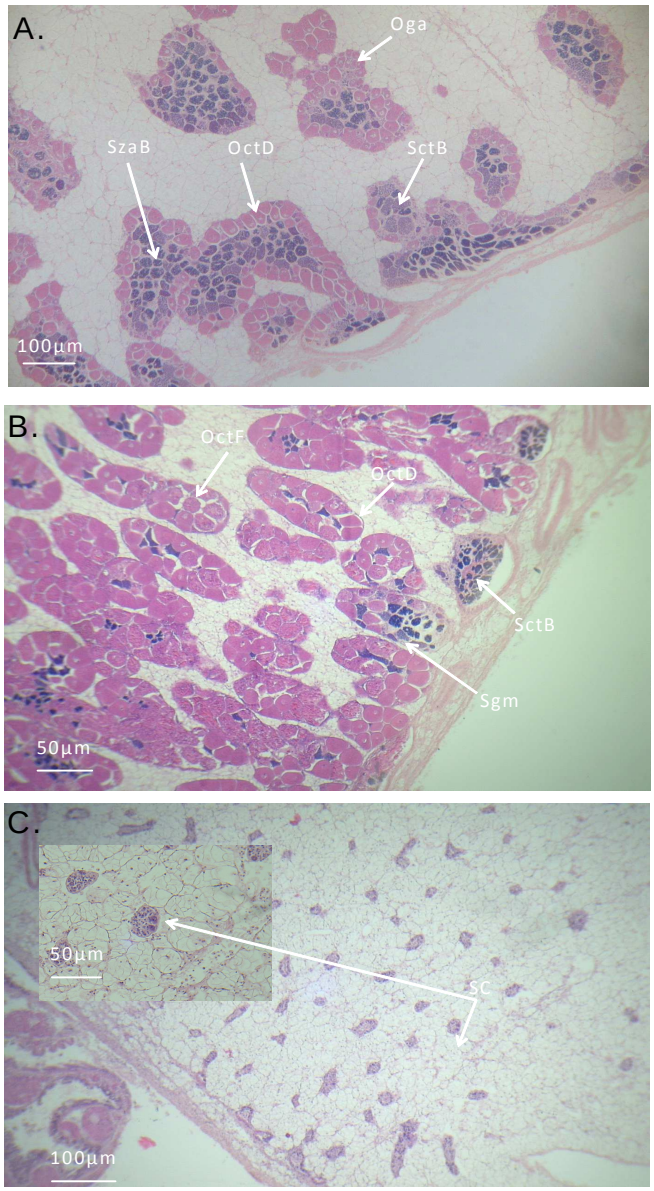

Figure 2.4 Micrographs of histological sections of *Ostrea angasi* gonad showing: **A**. Predominantly male hermaphrodite: with primarily spermatozoa balls (SzaB), few spermatocyte balls (SctB) and no spermatgonium (Sgm) present in combination with oogonia (Oga) arising from stem cells, developing oocytes (OctD) around periphery of the follicle and no free oocyte present. **B**. Predominantly female hermaphrodite: Free oocytes **(**OctF) developing oocytes (OctD) and no oogonia present, combined with spermatagonium and spermatocyte balls and no spermatozoa balls present. The term hermaphrodite is used to describe both gametes present in the gonad at the same time; they are not considered to true functional hermaphrodites. **C.** Non-defined stage of gametogenesis: no discernible

2.3 Results

2.3.1 Within estuary comparison

For all estuaries sampled there were no significant correlations between CI and temperature or salinity for the previous or -2 months (previous month: n=10, temperature r= 0.55 to -0.26, salinity r= 0.28 to –0.07, P>0.05; -2 month: n=10 temperature r=0.46 to -0.31, salinity r=0.11 to -0.41). The frequency of brooding oysters was not correlated with temperature or salinity from the previous or -2 months (brooding oysters: previous month: temperature n=10, r=-0.29 to 0.20, salinity n=10, r=-0.2 to 0.60; -2 month: temperature n=10, r=-0.28 to -0.09, salinity n=10, r=0.61 to -0.19). For oysters sampled from Narooma CI negatively correlated with proportion of brooding oysters from the previous month (r=-0.69. n=11, P=0.019). Condition index of oysters sampled from the previous or -2 months did not correlate with proportion of brooding oysters in the other estuaries (previous month: n=11, r=0.19 to -0.19; -2 month: n=10, 0.16 to -0.59). For all estuaries sampled CI of oysters sampled in the previous or -2 month was not correlated with the number of larvae produced (previous month: n=11, r=0.47 to -0.4; -2 month: n=10, r=0.41 to -0.28).

2.3.2 Laurieton.

Water temperature varied from 17^{o}C in May to 26.4^{o}C in February and salinity ranged from a minimum of 30.3 in July to a maximum of 33.3 in June (Fig. 2.5A). The average CI varied significantly among the months sampled (F_{month}=3.59, df 11, 348, P<0.001) and was greatest in March and smallest in January (Fig. 2.5B). Oysters sampled in January had a CI 17.5% less than oysters sampled in March, but they were not significantly different than oysters sampled in the other months. Oysters sampled in August had a CI significantly smaller than oysters sampled in October and March.

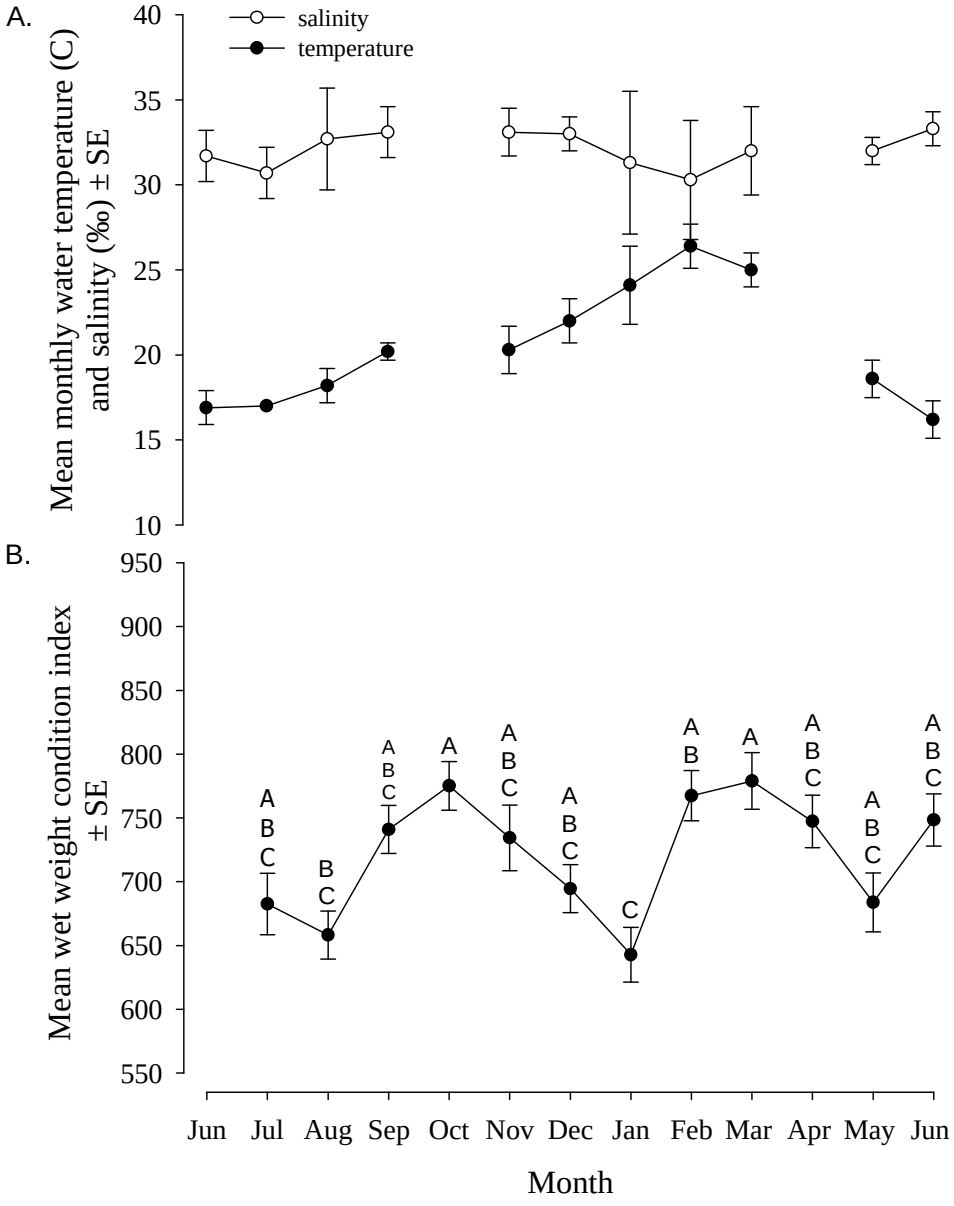

Figure 2.5 A. Mean monthly water temperature, salinity and **B**. Mean monthly wet weight condition index from *Ostrea angasi* collected from Camden Haven River, Laurieton, NSW. Means that are not significantly different from one another have the same letter.

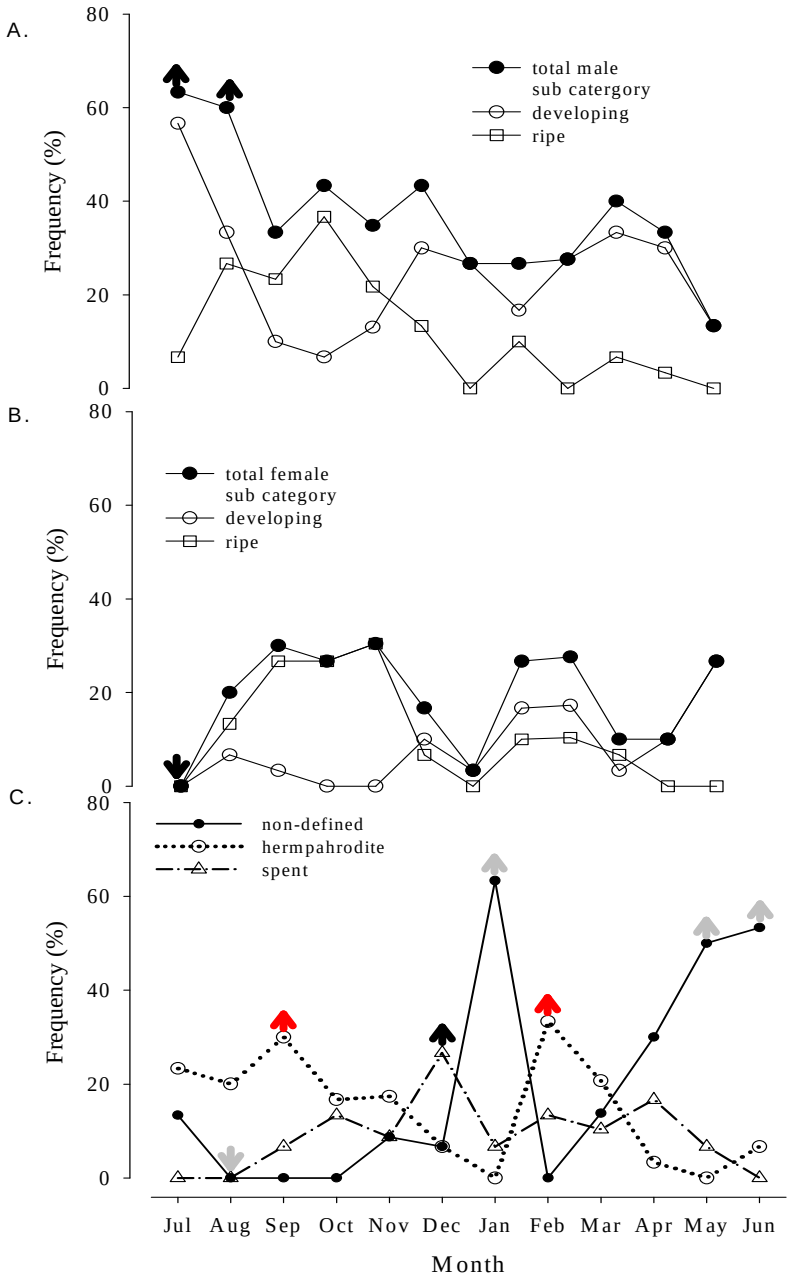

Figure 2.6 Percentage frequency of histological determined phases of the total *Ostrea angasi* gamete development of **A**. Total male and subcategory developing and ripe (black arrow) **B.** Total female and subcategory developing and ripe & **C**. Non-defined (blue arrow), hermaphrodite (red arrow) and spent (black arrow) from *Ostrea angasi* collected from Camden Haven River, Laurieton, NSW. Arrows indicate frequencies were more or less than expected.

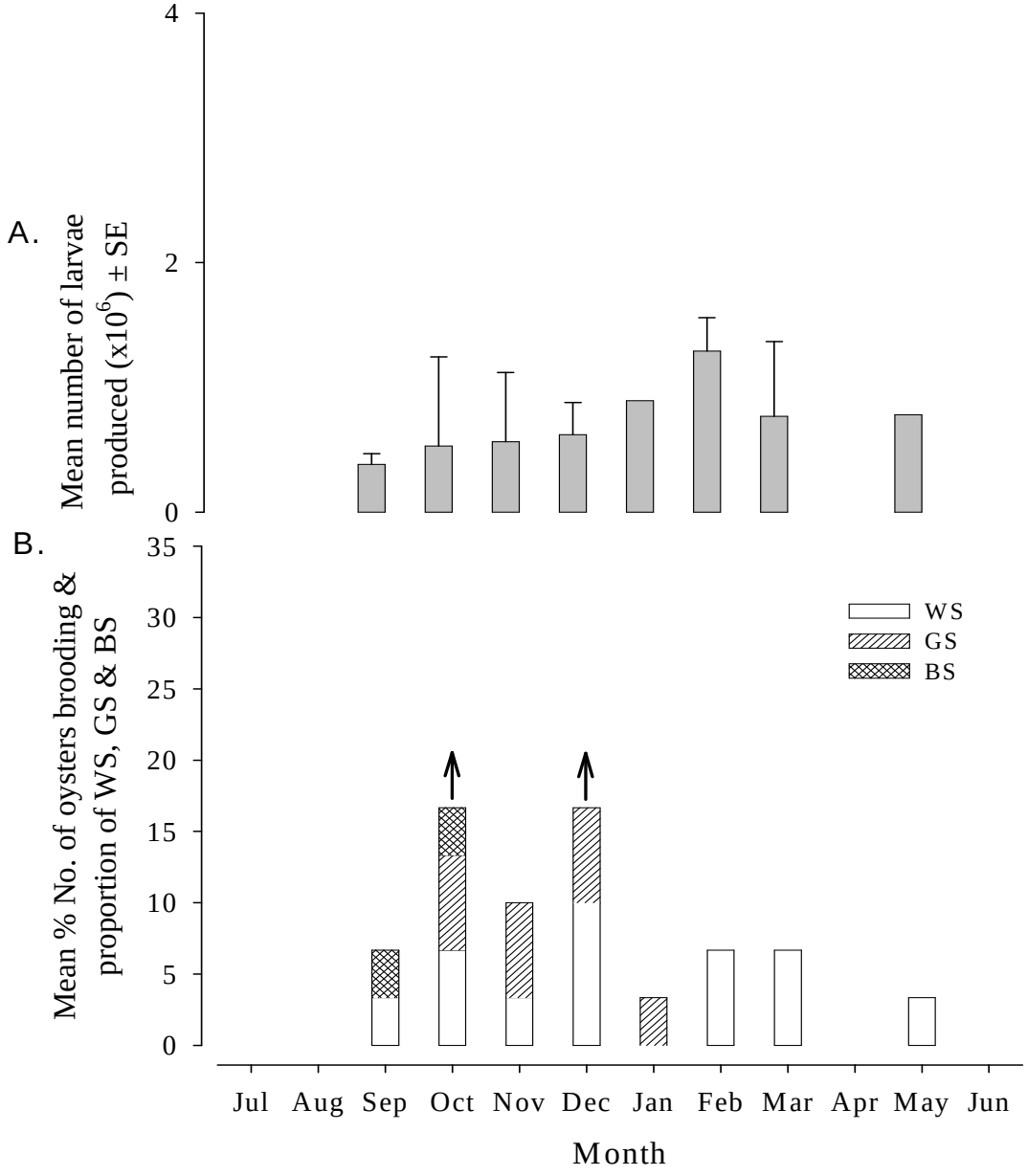

Figure 2.7 A. Mean monthly number of larvae per brooding female and **B**. Percentage of brooding oysters with the proportion of larval developmental stages of: white sick (WS), grey sick (GS) and black sick (BS) from *Ostrea angasi* collected from Camden Haven River, Laurieton, NSW. Note that months with no data were when no brooding females were found among the 30 oysters sampled. Arrows indicate the direction of departure of observed frequency of brooding oysters from expected frequencies.

The proportion of oysters in each of the five gametogenic phases varied over the 12 months sampled (χ^2 = 203, df 44, P<0.001). More than expected male oysters were present in July and August (Fig. 2.6A) and fewer than expected female oysters were present in July (Fig. 2.6B). Fewer than expected non-defined oysters were present in August, September, October and February and more than expected non-defined oysters were sampled in January, May and June (Fig. 2.6C). More than expected hermaphrodites were present in September and February, less than expected hermaphrodite oysters were present in January and February and more than expected spent oysters were present in December.

The CI of oysters sampled in -2 month was negatively correlated with the proportion of hermaphrodite oysters and positively correlated with the proportion of spent & non-defined oysters (r=-0.72, n=12, p=0.02 and r=0.79, n=12, p=0.002 respectively). The proportion of oysters gametogenic phases did not correlate with temperature or salinity for the previous or -2 month (previous month: temperature n=10 r=0.52 to 0.42, salinity n=10 r=0.38 to -0.42; -2 month: temperature n=10 r=0.58 to -0.50, salinity n=10 r=0.46 to -0.62). The proportion of hermaphrodites was positively correlated with proportion of spent oysters from the previous month and spent & non-defined from the following month (r=0.63, n=11, P=0.038 and r=0.63, n=11, P=0.038 respectively). The proportion of female hermaphrodites positively correlated with the proportion of ripe male oysters from previous month (r=0.84, n=11, P=0.001) and the proportion of hermaphrodites positively correlated with the proportion of male oysters from the previous month (r=0.76, n=11, P=0.007).

The average number of larvae per oyster (larvae produced) did not differ among the eight months that brooding oysters were present (F_{month}=1.18, df 7, 13, P=0.38) (Fig. 2.7A), with black, grey and white sick larvae present (Fig. 2.7B). The proportion of brooding oysters varied significantly over the 12 months sampled (χ^2 = 17.9, df 11, P=0.019), with the greatest number found in October and December and no brooding oysters present in April, June, July, or August. The proportion of brooding oysters was positively correlated with the proportion of spent oysters in same month (r=0.79, n=12, P=0.002). Histological examination of the gonads of brooding oysters indicated 52% had male gametes present and the remainder were

non-defined. Estimates of the frequency of oysters brooding range from 1.59-3.50 times in the 12 month sampled, depending on the brooding period used (P=1.59 for 22 days brooding and P= 3.5 for 10 days brooding respectively).

2.3.3 Narooma

Water temperature varied from a minimum of 12.2^{o}C in June to a maximum of 23^{o}C in January and salinity ranged from a minimum of 29 in June to a maximum of 34.7 in February (Fig 2.8A). The average CI varied significantly among the months sampled (F_{month}=14.25, df 11, 347, P<0.001) was greatest in May but did not differ from oysters sampled in October, December, January, March, April or June (Fig 2.8B). Oysters sampled in November had a CI 28.1% smaller than oysters sampled in May and differed only from oysters sampled in August and July. Condition index of oysters sampled in September was significantly greater than oysters sampled in November but less those sampled in April or May.

The proportion of oysters in each of the gametogenic phases varied over the 12 months sampled (χ^2 = 122.05, df 44, P<0.001). The proportion of the males and females did not depart from the expected frequencies for the 12 month sampled (Fig. 2.9A & B). Fewer than expected non-defined oysters were sampled in September and more than expected non-defined oysters were sampled in May (Fig. 2.9C). Fewer than expected hermaphrodites were sampled in May and more than expected spent oysters were sampled in December.

The proportion of hermaphrodites positively correlated with temperature from previous month and spent oysters negatively correlated with CI from the previous month (r=0.66, n=11, P=0.028 and r=-0.82, n=11, P=0.002). The proportion of developing males negatively correlated with the proportion of hermaphrodites from previous month (r=-0.63, n=11, P=0.036). The proportion male hermaphrodites positively correlated with the proportion brooding oysters from following month and negatively correlated with developing male oysters from previous month (r=0.65, n=11, P= 0.029 and r= -0.63, n=11, P= 0.036 respectively).

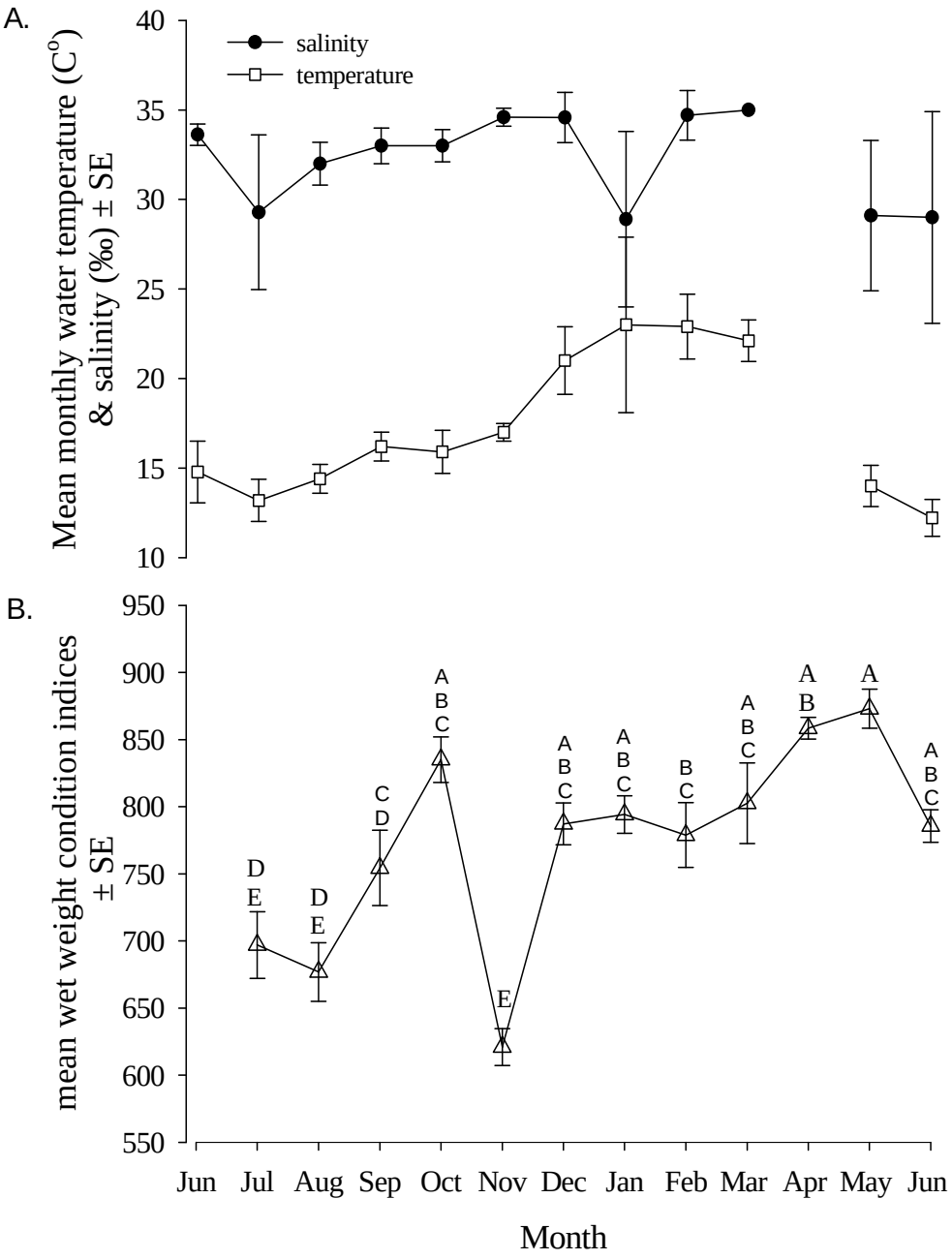

Figure 2.8 A. Mean monthly water temperature, and salinity & **B**. Mean monthly wet weight condition index of *Ostrea angasi* collected from Wagonga Inlet, Narooma, NSW. Means that are not significantly different from one another have the same letter.

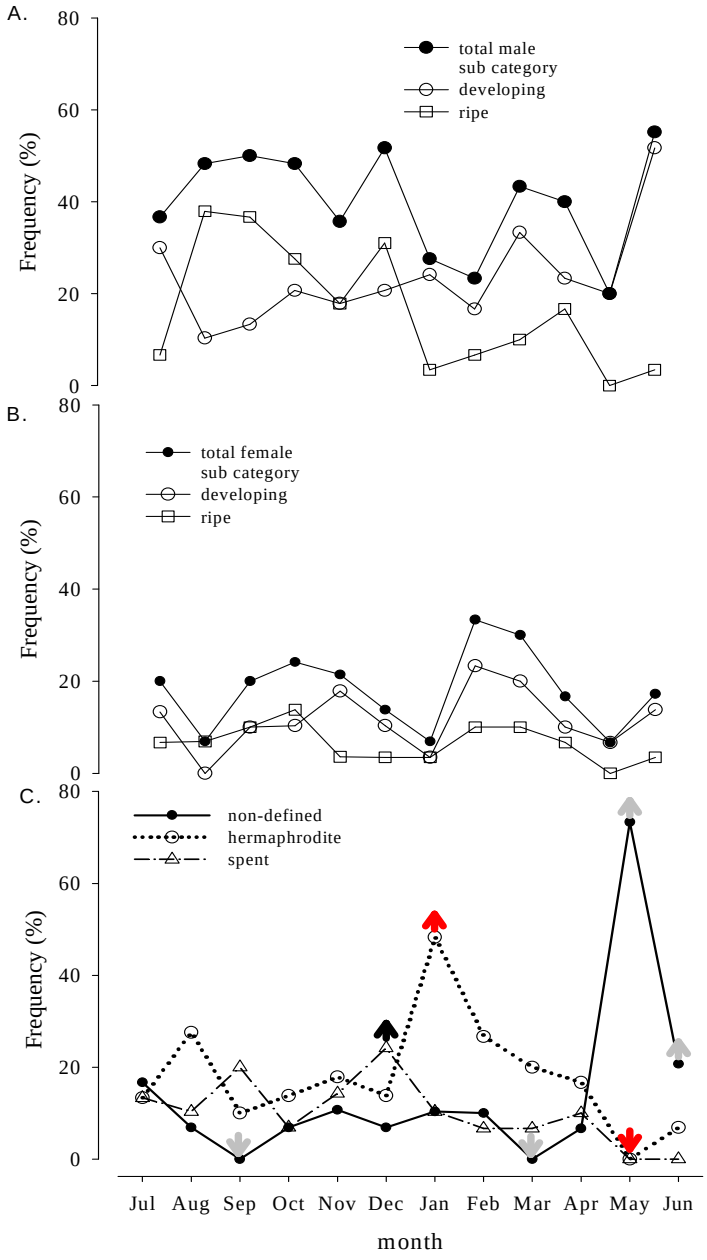

Figure 2.9 Percentage frequency of histological determined phases of the total *Ostrea angasi* gamete development of **A**. Total male and subcategory developing and ripe (black arrow) **B**. Total female and subcategory developing and ripe & **C**. Non-defined (blue arrow), hermaphrodite (red arrow) and spent (black arrow) from *Ostrea angasi* collected from Wagonga Inlet, Narooma, NSW. Arrows indicate frequencies were more or less than expected.

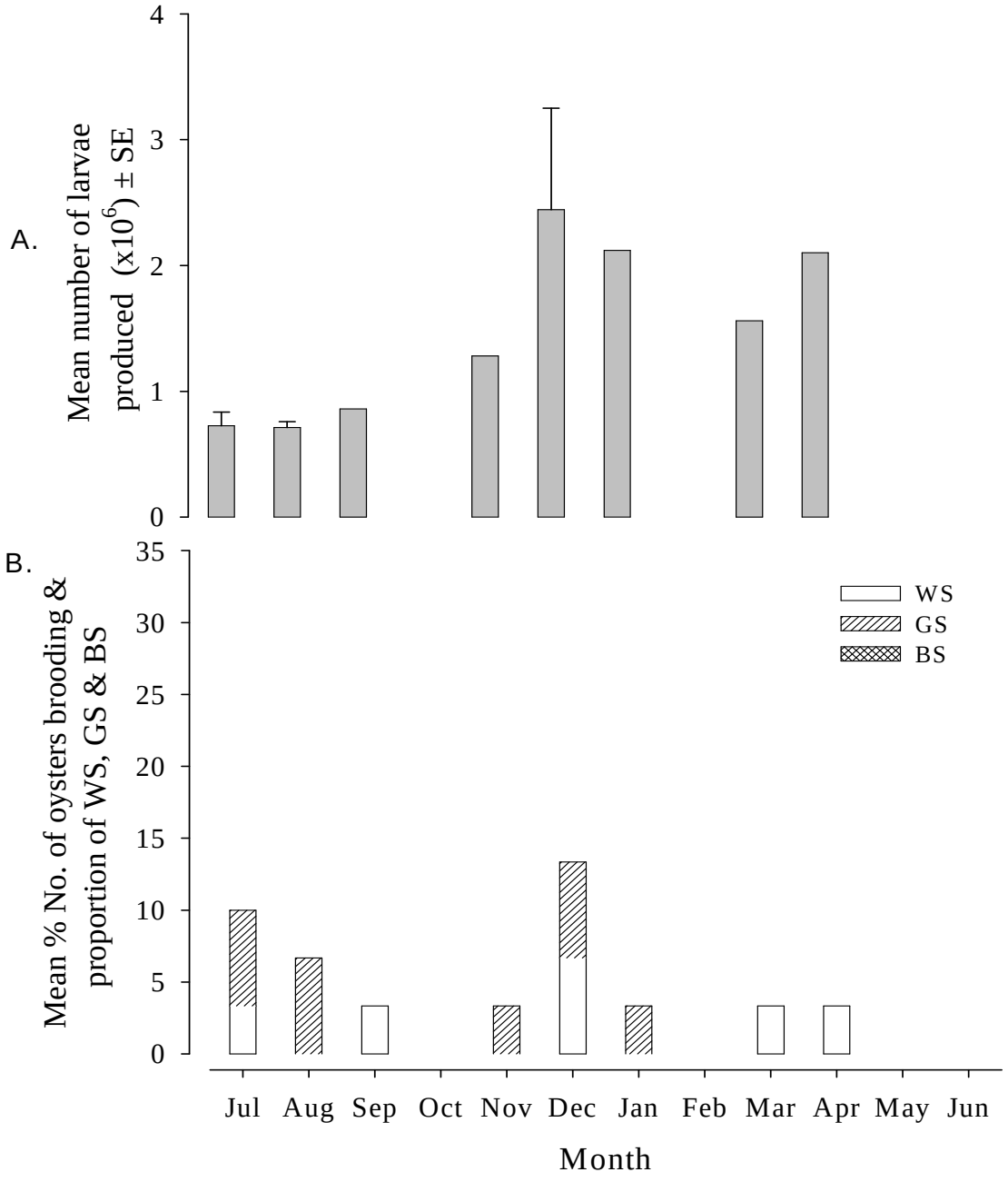

Figure 2.10 **A**. Mean monthly number of larvae per female & **B**. Mean monthly percentage of brooding oysters with the proportion of larval developmental stages of: white sick (WS), grey sick (GS) and black sick (BS) from *Ostrea angasi* collected from Wagonga Inlet, Narooma, NSW. Note that months with no data were when no brooding females were found among the 30 oysters sampled.

The number of larvae produced did not differ among the eight months that brooding oysters were present (F_{month}=2.66, df 7, 7, P=0.11) (Fig 2.10A), with only grey and white sick larvae observed (Fig. 2.10B). The proportion of brooding oysters did not depart from the expected frequencies over the 12 months sampled (χ^2 = 11.26, df 11, P=0.20). Brooding oysters were not present in October, February, May or June. The proportion of brooding oysters positively correlated with the spent oysters from the same month (r=0.79, n=12, P=0.002). Histological examination of the gonads of brooding oysters indicated 47% had male gametes present and the remainder were non-defined. Estimates of the frequency of oysters brooding range from 1.06-2.33 times in the 12 months sampled, depending on the brooding period used (P=1.06 for 22 days brooding and P= 2.33 for 10 days brooding respectively).

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2.3.4 Bermagui

Water temperature varied from a minimum of 14.2^{o}C in May to a maximum of 22^{o}C in January and salinity ranged from a minimum of 33.8 in May to a maximum of 36 in March (Fig. 2.11A). The average CI varied significantly among the months sampled (F_{month}=8.0, df 10, 318, P<0.001) and was greatest in December but did not differ from oysters sampled in October, January, February, April, May and June (Fig. 2.11B). Oysters sampled in August had a CI 21% smaller than oysters sampled in December, but did not differ from oysters sampled in July, September, October or November. The CI of oysters sampled in July was significantly smaller than oysters sampled in December, January, May or June. The CI of oysters sampled in April was significantly greater than oysters sampled in July, August or November. The CI of oysters sampled in February was only significantly greater than oysters sampled in August or September.

The proportion of oysters in each of the gametogenic phases varied over the 12 months sampled (χ^2 = 100.34, df=40, P<0.001). More than expected males were sampled in December (Fig. 2.12A) and more than expected females were sampled in February (Fig 2.12. B). More than expected non-defined oysters were sampled in July and fewer than expected were sampled in October, February and June. The proportion of hermaphrodites did not depart from expected numbers for the 12 months sampled. More than expected spent oysters were sampled in December (Fig. 2.12 C). The proportion of males negatively correlated with temperature from the previous month (r=-0.67, n=11, P=0.023). The proportion of hermaphrodites positively correlated with ripe females from following month (r=0.68, n=9, P=0.045). The proportion of spent oysters positively correlated with female hermaphrodites

from the previous month (r=0.70, n=9, P=0.035). The proportion of hermaphrodites positively correlated with females from the previous month (r= 0.73, n=9, p=0.025) and the proportion of female hermaphrodites positively correlated with spent oysters from the previous month (r=0.70, n=9, P=0.35).

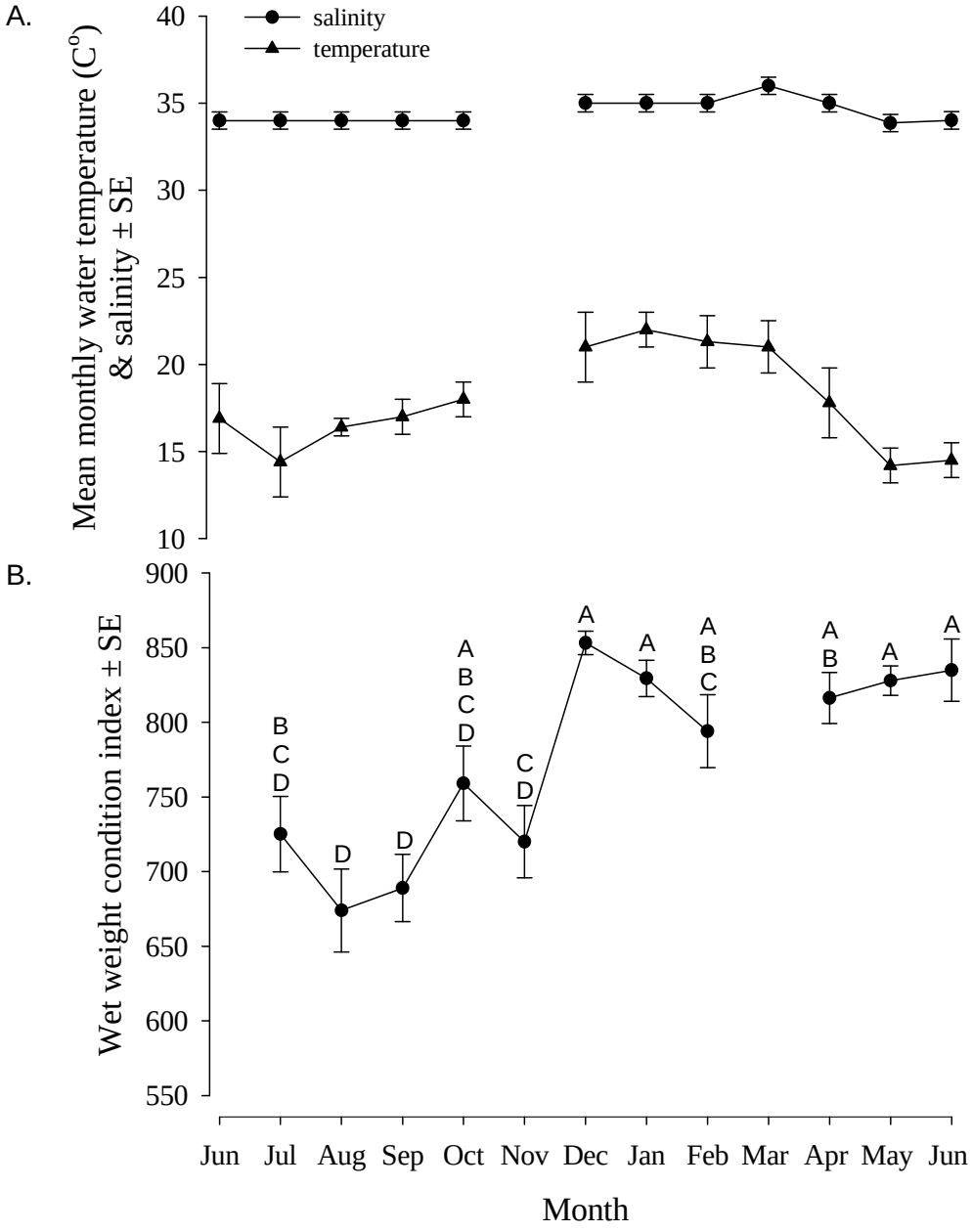

Figure 2.11 A. Mean monthly water temperature and salinity & **B**. Mean monthly wet weight condition index from *Ostrea angasi* collected from Bermagui River, Bermagui, NSW. Means that are not significantly different from one another have the same letter.

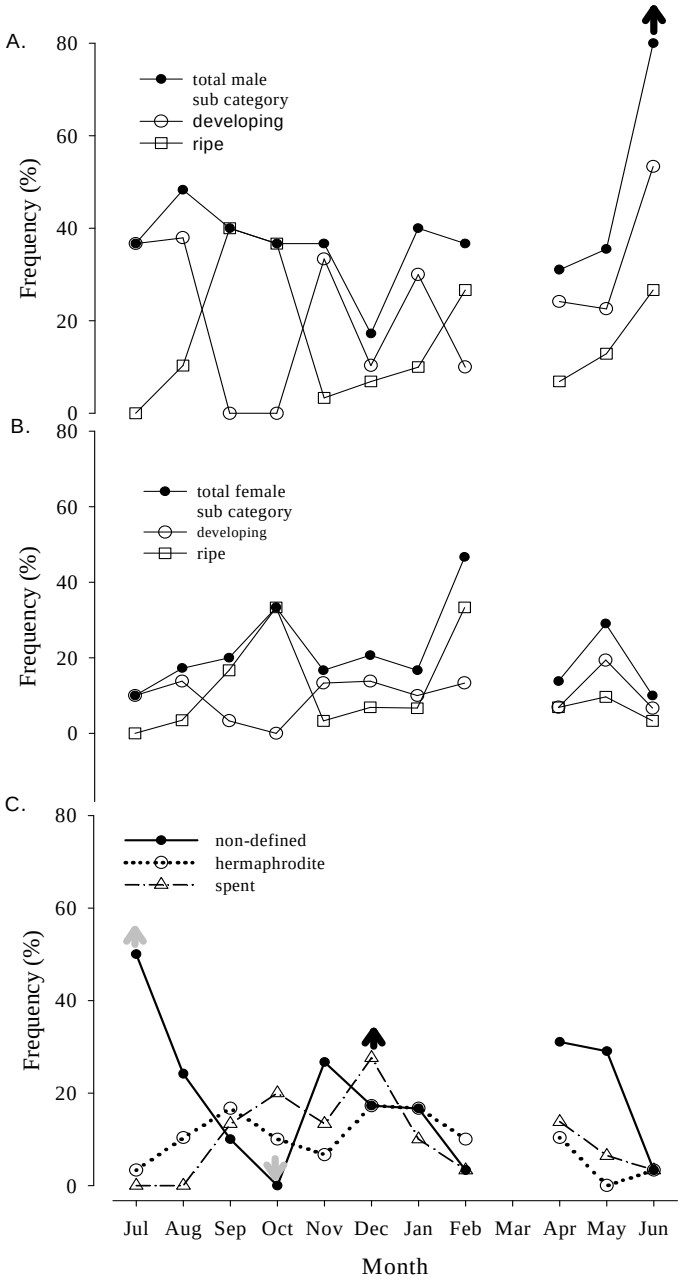

Figure 2.12 Percentage frequency of histological determined phases of the total *Ostrea angasi* gamete development of **A**. Total male and subcategory developing and ripe (black arrow) **B**. Total female and subcategory developing and ripe & **C**. Non-defined (blue arrow), hermaphrodite (red arrow) and spent (black arrow) from *Ostrea angasi* collected from Bermagui River, Bermagui, NSW. Arrows indicate frequencies were more or less than expected.

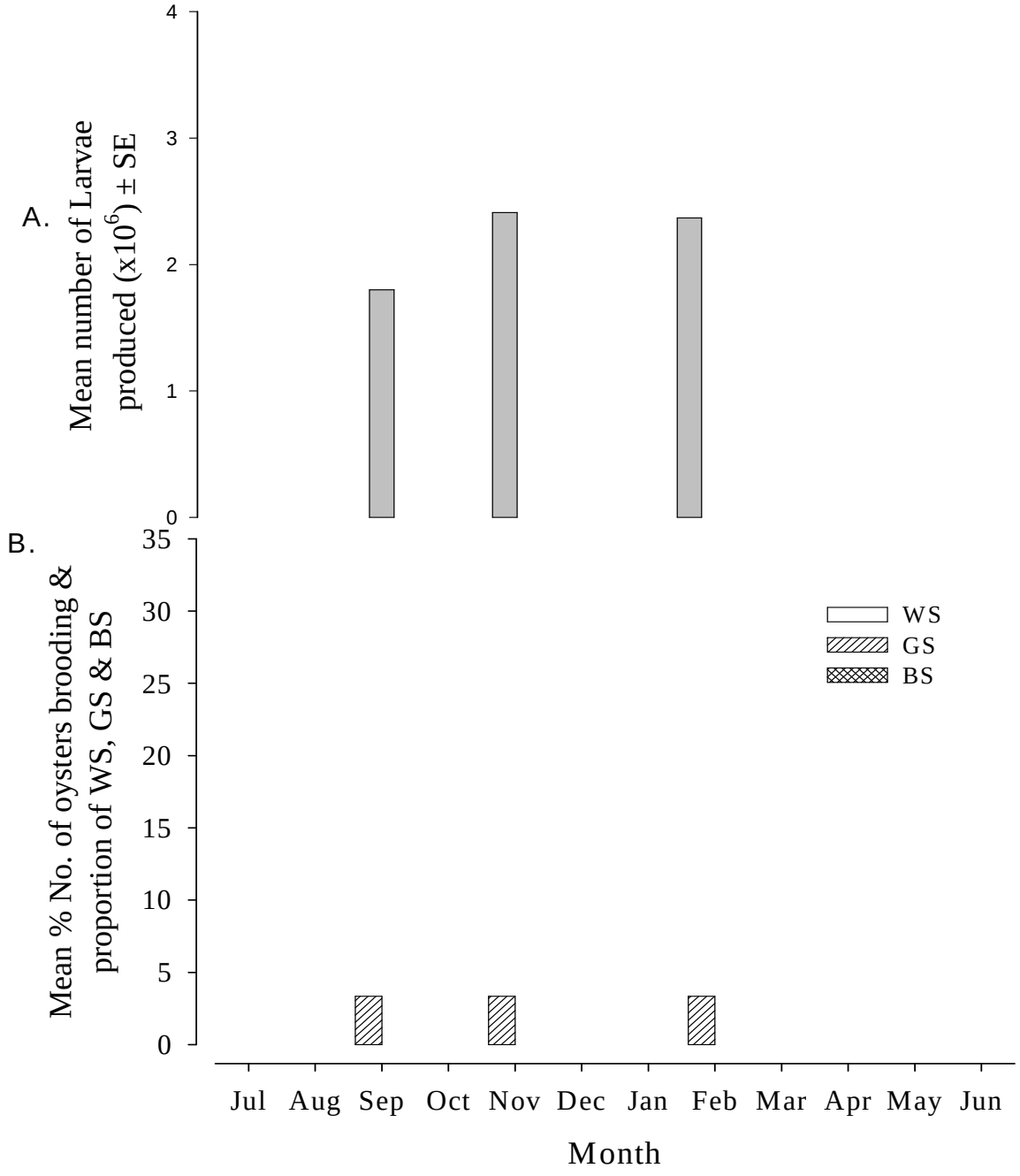

Figure 2.13 A. Mean monthly number of larvae produced & **B**. Mean percentage of oysters brooding and proportion of larval developmental stages of: white sick (WS), grey sick (GS) and black sick (BS) from *Ostrea angasi* collected from Bermagui River, Bermagui, NSW. Note that months with no data were when no brooding females were found among the 30 individuals sampled.

No comparison was made between the number of larvae produced and month sampled as only three oysters were found brooding larvae in three different months (Fig. 2.13A). Brooding oysters were present in only three months of the 12 months sampled, September, November and February and on all occasions grey sic larvae were observed (Fig. 2.13B). The proportion of oysters brooding was consistent for each of the 3 months. The proportion of brooding oysters positively correlated with spent oysters from the same month (r=0.83, n=9, P=0.006). Histological examination of the gonads of brooding oysters indicated 33% had male gametes present and the remainder were non-defined. Estimates of the frequency of oysters brooding range from 1.06-2.33 times in the 12 months sampled, depending on the brooding period used (P=1.06 for 22 days brooding and P= 2.33 for 10 days brooding respectively). Estimates of the frequency of oysters brooding range from 0.23-0.5 times in the 12 months sampled, depending on the brooding period used (P=0.227 for 21 days brooding and P= 0.5 for 10 days brooding).

2.3.5 Merimbula

Water temperature varied from a minimum of 12^{o}C in June to maximum of 21^{o}C in January and salinity ranged from a minimum of 28.9 in January to a maximum of 35 in March (Fig. 2.14A). The average CI varied significantly among the months sampled (F_{month}=11.93, df 11, 348, P<0.001) and was greatest in June, but only greater than July and August (Fig. 2.14B). Oysters sampled in July had a CI 29.3% smaller than oysters sampled in June and were significantly smaller than oysters sampled in the other months. Oysters sampled in August had a CI significantly smaller than oysters sampled in all months, excepting oysters sampled in July, September, November and May.

The proportion of oysters in each gametogenic phase varied over the 12 months sampled (χ^2 = 150.23, df=44, P<0.001). More than expected males were sampled in June and fewer than expected sampled in January (Fig. 2.15A). More than expected females were sampled in November and February and fewer than expected sampled in May and June (Fig. 2.15B). More than expected non-defined oysters were sampled in July, January and May and fewer than expected sampled in August, September and December (Fig. 2.15C). More than expected hermaphrodites were sampled in August and more than expected spent oysters were sampled in December and April (Table 2B). The proportion of males negatively correlated with temperature and the proportion of females positively correlated with temperature. The proportion of hermaphrodites negatively correlated with the proportion of brooding oysters

from the previous month (r=-0.68, n=1, P=0.022). The proportion of female hermaphrodites positively correlated with the proportion of spent oysters from previous month (r=0.73, n=11, P=0.011) and the proportion of equal hermaphrodites negatively correlated with the proportion of developing females from the following month (r=-0.74, n=11, P=0.01).

The number of larvae produced differed significantly among the nine months that brooding oysters were present (F_{month}=24.81, df 8, 7, P<0.001) (Fig. 2.16A), with black, grey and white sick larvae observed on 5, 7 and 9 occasions, respectively (Fig. 2.16B). The number of larvae produced was greatest from oysters sampled in May, though the number larvae produced in this month did no differ from that produced per oyster in February, March, and April. The number of larvae produced from oysters sampled in November was approximately 90% lower than that produced per oyster sampled in May and was significantly lower than the number of larvae produced per oyster sampled from all other months when brooding oysters were present. The number of larvae produced from oysters sampled in April did not differ from the number of larvae produced from oysters sampled in July but was greater than the number of larvae produced from oysters sampled in December. There was no difference in the number of larvae produced from oysters sampled in September, October and December. The proportion of brooding oysters did not depart from the expected frequencies over the 12 months sampled (χ^2 = 9.69, df 11, P=0.45). No brooding oysters were observed in August, January or June. The proportion of brooding oysters negatively correlated with CI from the same month (r=0.62, n=12, P=0.032). Histological examination of the gonads of brooding oysters indicated 44% had male gametes present and the remainder were non-defined. Estimates of the frequency of oysters brooding range from 1.21-2.66 times in the 12 months sampled, depending on the brooding period used (P=1. 21 for 21 days brooding and P= 2.66 for 10 days brooding

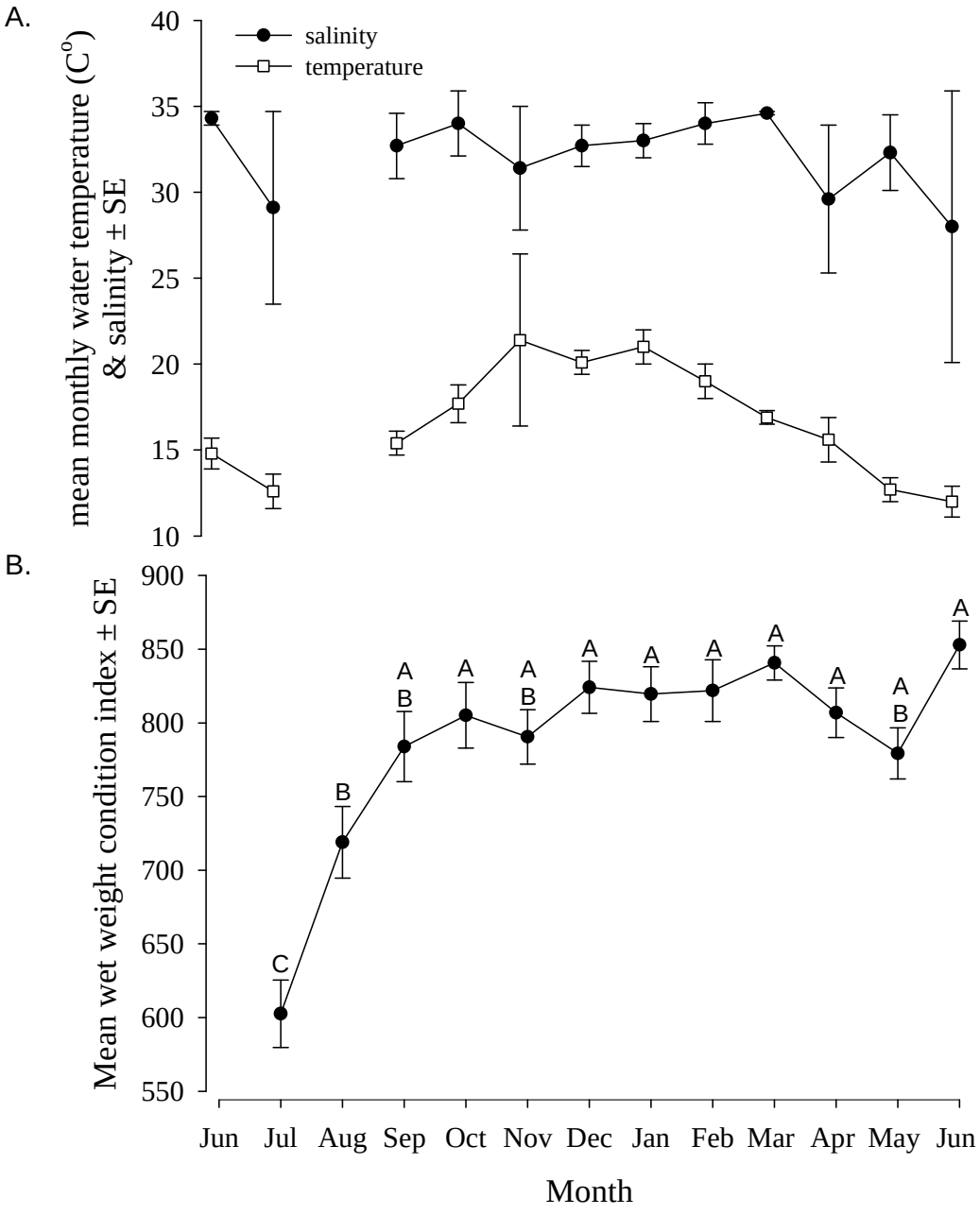

Figure 2.14 A. Mena monthly water temperature and salinity & **B**. Mean monthly wet weight condition index of *Ostrea angasi* collected from Merimbula Lake, Merimbula, NSW. Means that are not significantly different from one another have the same letter

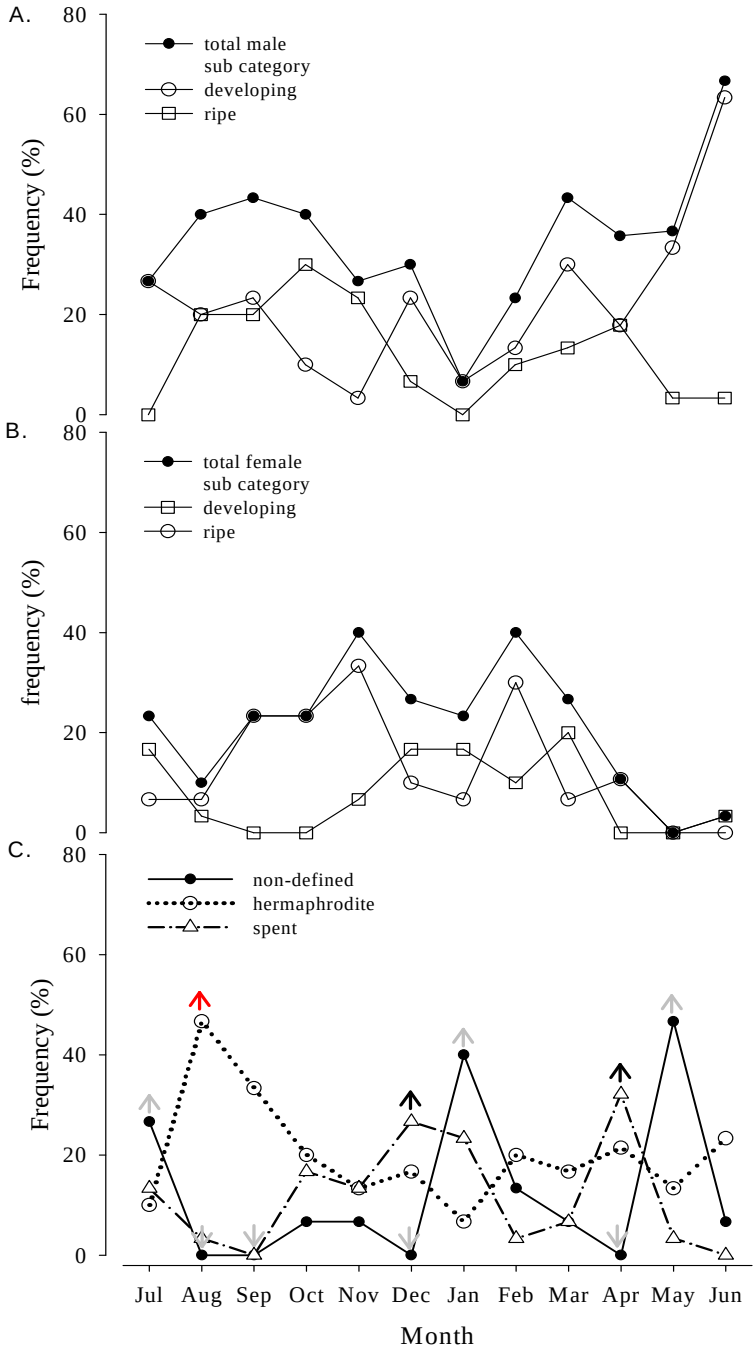

Figure 2.15 Percentage frequency of histological determined phases of the total *Ostrea angasi* gamete development of **A**. Total male: subcategory developing and ripe (black arrow) **B**. Total female: subcategory developing and ripe & **C**. Non-defined (blue arrow), hermaphrodite (red arrow) and spent (black arrow) from *Ostrea angasi* collected from Merimbula Lake, Merimbula, NSW. Arrows indicate frequencies were more or less than expected.

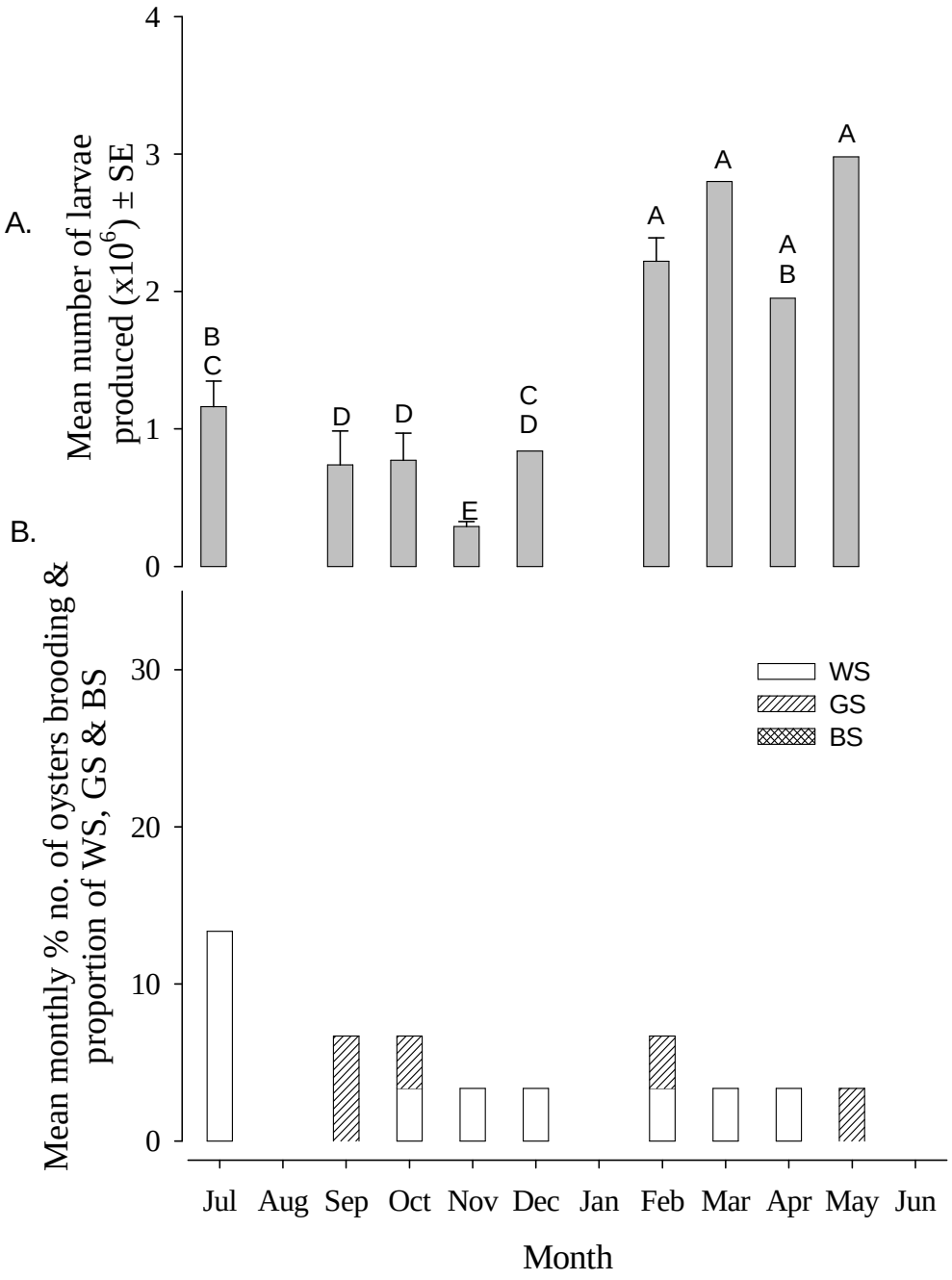

Figure 2.16 A. Mean monthly number of larvae produced & **B**. Mean monthly percentage of oysters brooding and proportion of larval developmental stages of: white sick (WS), grey sick (GS) and black sick (BS) from *Ostrea angasi* collected from Merimbula Lake, Merimbula, NSW. Note that months with no data were when no brooding females were found among the 30 oysters sampled. Means that are not significantly different from one another have the same letter.

2.3.6 Among estuary comparison

The average number of larvae produced per oyster differed significantly among the four estuaries ($F_{estuary}$ =8.31, df 3, 51, P<0.001). Brooding oysters collected from Bermagui had on average 50% more larvae than brooding oysters from the other three estuaries (Fig. 2.17A). The size range of brooding oysters was 49-95mm, with significantly smaller (average 12.33 mm smaller) oysters found brooding from Laurieton (F=14.02, df 3, 51 P<0.001). Brooding oyster were gametogenically assessed as either non-defined or male and there was no difference among estuaries in frequency gametogenic phases of brooding oysters.

Both the mean CI of oysters and the percentage of brooding oysters differed significantly among the estuaries (F_{CI}=17.42, df 3, 1404, P<0.001; $F_{brooding}$=3.26, df 3, 44, P=0.03). Oysters from Laurieton had an average CI 6% smaller than oysters sampled from the three southern estuaries (Fig 2.17B), but the population of oysters at Laurieton had a 7 fold greater average percentage of oysters brooding than the Bermagui population. For the 12 months sampled combined the frequency of individuals in each of the gametogenic phases differed among estuaries (χ^2 =19.29, df 9, P=0.023). The proportion of hermaphrodites sampled from Bermagui was 14% less than the other estuaries and Merimbula had 14.3% more hermaphrodites compared with the other estuaries (Fig. 2.18). Among all estuaries, for the sampling period combined, the frequency of developing male oysters was greater than expected and the frequency of ripe male oysters was less than expected (Table 4). Oysters sampled from Merimbula had less than expected developing females and more than expected ripe females (Table 2.2).

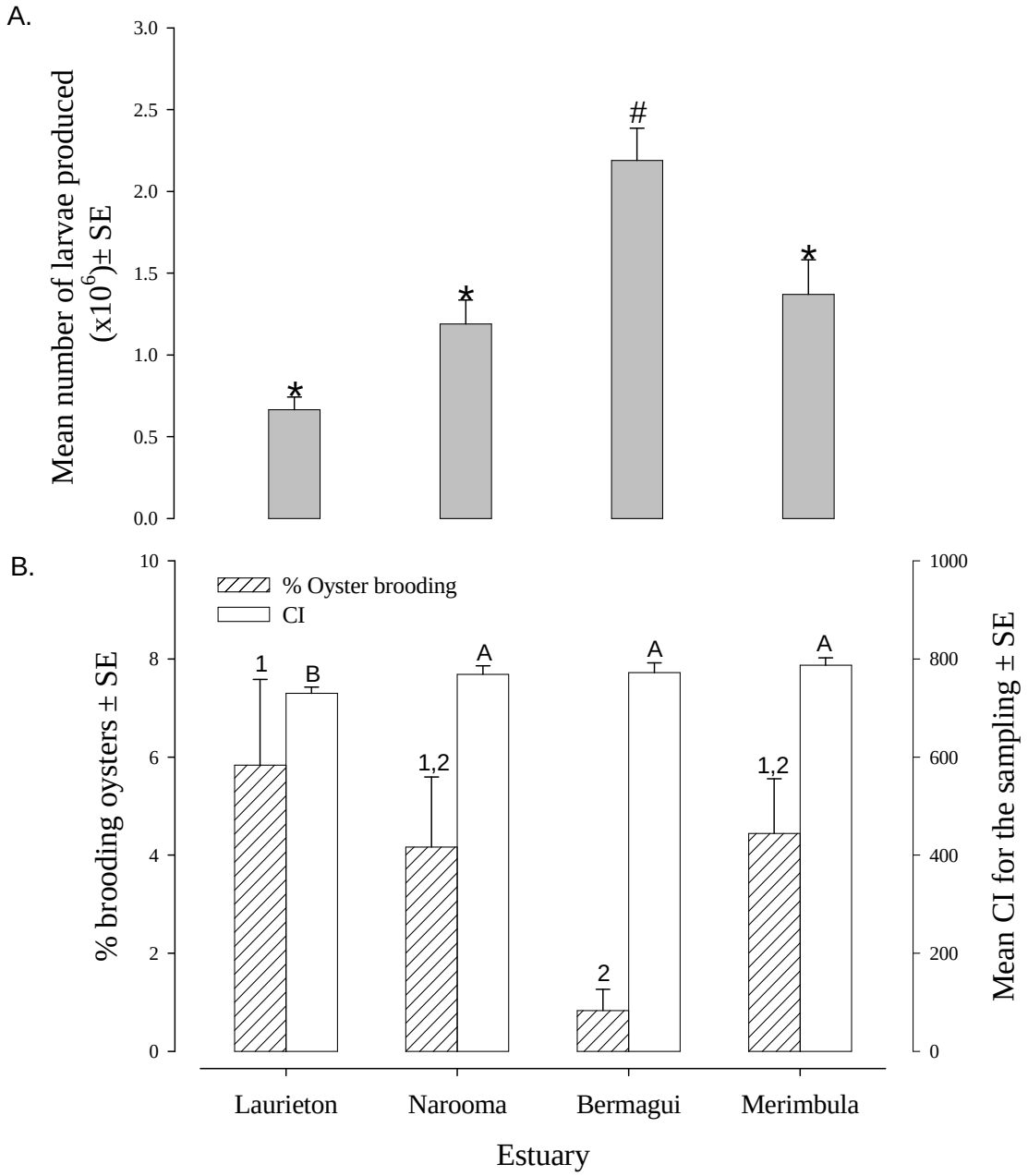

Figure 2.17 A. Mean number of *Ostrea angasi* larvae produced per oyster, **B**. Percentage of brooding oysters and wet weight condition index pooled across 12 months sampled for the four estuaries, Camden Haven River (Laurieton), Wagonga Inlet (Narooma), Bermagui River (Bermagui) and Merimbula Lake (Merimbula). Means with the same letter, number or symbol are not significantly different from one another.

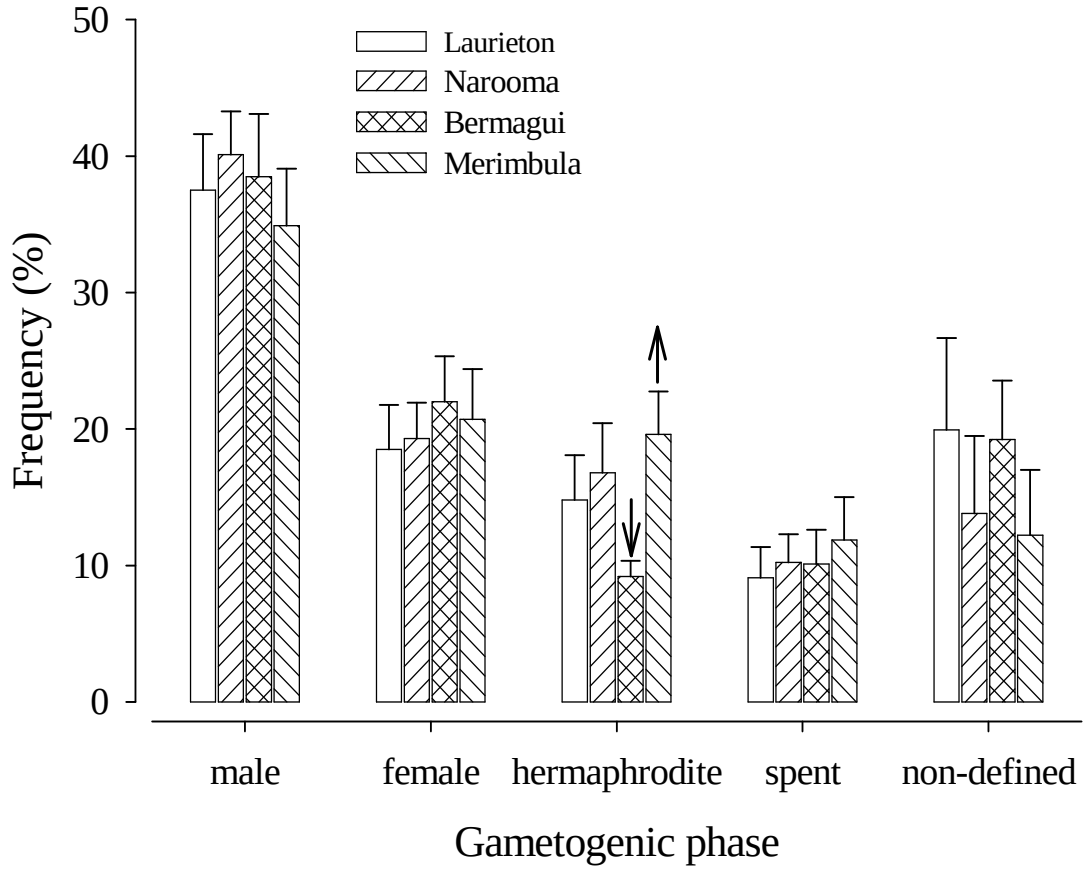

Figure 2.18 Percentage frequency of male, female, hermaphrodite, spent and non-defined gametogenic phases for the entire sampling period combined for the four estuaries in NSW, Camden Haven River (Laurieton), Wagonga Inlet (Narooma), Bermagui River (Bermagui) and Merimbula Lake (Merimbula). Arrows indicate the direction of departure of frequency of gametogenic phase from expected frequencies.

Table 2.2 Chi-squared values for the frequency of histologically determined gametogenic phase of developing and ripe male or females oysters sampled from four estuaries in NSW, Camden Haven River (Laurieton), Wagonga Inlet (Narooma), Bermagui River (Bermagui) and Merimbula Lake (Merimbula), for a 12 month sampling period.

Estuary	Gametogenic Phase	Chi-square	developing	ripe
Laurieton	Male	χ^2 =18.93, df 1, P<0.001	more than expected	less than expected
	Female	χ^2 =1.62, df 1, P= 0.251	-	-
Narooma	Male	χ^2 =6.51, df 1, P=0.014	more than expected	less than expected
	Female	χ^2 =2.82, df 1, P= 0.125	-	-
Bermagui	Male	χ^2 =5.56, df 1, P=0.024	more than expected	less than expected
	Female	χ^2 =0.41, df 1, P=0.611	-	-
Merimbula	Male	χ^2 =13.44, df 1, P<0.001	more than expected	less than expected
	Female	χ^2 =5.43, df 1, P<0.028	less than expected	more than expected

2.4 Discussion

Over the geographical range sampled in NSW, brooding adults were available for eleven months of the year and even in the most southern estuary, Merimbula, brooding oysters were observed for nine of the 12 months sampled. Within the estuaries sampled in NSW, no evidence of a seasonal peak in the frequency of brooding adults was observed. Spawning events in Laurieton, Narooma and Merimbula were asynchronous, given that for a location and month, the age of the larval broods differed among females. Too few brooding oysters were found from Bermagui to comment on the synchronicity of spawning events in this

estuary. The spawning events of *O. angasi* in southern Australia is temporally restricted and broadly constrained to the warmer months of the year (Dix, 1976; O'Sullivan, 1980). At the southern extent of the species range in Tasmania the breeding season for *O. angasi* is November to February (Sumner, 1972). Further to the north, in Port Phillip Bay, Victoria, the breeding season extends from October to February (Hickman and O'Meley, 1988a), whereas, in West Lakes, South Australia the breeding season of *O. angasi* is from October until March (O'Sullivan, 1980). However, such strong seasonal restriction in spawning was not evident in NSW with brooding oysters observed year round. While the farm environment may encourage oyster condition, growth rates, and potentially reproduction (Haven, 1960; Toro et al., 1995), this is unlikely to be the controlling factor as in South Australia the seasonal pattern of reproduction in farmed oysters (Xiaoxu Li, pers comm, 2015) is similar to that observed in wild populations (O'Sullivan, 1980). Genetic differences within populations can influence reproduction; however, the lack of genetic divergence of *O. angasi* in Australia does not support this hypothesis either (Hurwood et al., 2005). All farmed oyster examined were a mix of hatchery produced stock from the PSFI.

The absence of a strong seasonal pattern in the spawning of *O. angasi* populations in NSW compared to more southern populations may be linked to seawater temperature. While it has been concluded that spawning occurs at water temperatures >18^{o}C (O'Sullivan, 1980), given brooding oysters were found in mid-winter at Merimbula when water temperatures were 13^{o}C there is no evidence to suggest that gametogenesis and spawning is halted at water temperatures <18^{o}C. Gametogenesis and spawning of *O. angasi* continued at temperatures as high as 25^{o}C, the warmest mean monthly temperature recorded from the most northern estuary sampled, where brooding oysters were found. Additionally, the minimum temperature required to halt gametogenesis of *O. angasi* appears to be well below 18^{o}C as brooding oysters were found within NSW estuaries at temperatures as low as 13^{o}C.

Estimates of the proportion of farmed *O. angasi* brooding larvae over a season in NSW were relatively high compared to that reported in a wild population in South Australia, between 0.91 to 1.33 times during the shorter breeding season (O'Sullivan, 1980: though a minimum brooding period of 15 days was used as opposed 10 days for this study). In the 12 months sampled, oysters >49 mm were estimated to brood larvae from 1.59 to 3.50 times year^{-1} at Laurieton in the north to 1.2 to 2.66 times year^{-1} at Merimbula in the south. These greater estimates of number of times *O. angasi* can brood over a 12 month period in NSW waters

may in part be due to water temperatures not falling low enough to halt gametogenesis (Tranter, 1958). In South Australia a decrease in spawning activity of *O. angasi* occurred even though water temperatures were still favourable for reproduction (O'Sullivan, 1980) and may indicate that factors other than water temperature is effecting reproduction in southern populations.

The temporal changes in the CI of *O. angasi* in the four estuaries showed no evidence of being associated with seasonal changes in water temperature and salinity. Though dry CI have been shown to better reflect the gametogenic cycle (Lucas and Beinger, 1985), wet weight CI have been shown to follow dry weight CI though greater variability in wet weight CI was noted in *Panopea Zelandica* (Gribben et al., 2004). Condition indices are predictors of reproduction and spawning in other flat oyster species (Walne, 1964), and CI increases prior to spawning for *O. edulis* (Ruiz et al., 1992; Cano, et al., 1997). However no association between CI and spawning events were found. It is possible that spawning activity has influenced CI and shell length of oysters, given Laurieton had the greater frequency of brooding oysters, which were also significantly smaller than oysters in the other estuaries sampled. Alternatively, *O. angasi* may mature at a smaller size in the warmer northern waters at Laurieton (Angilletta et al., 2004). Regardless, the greater frequency of reproductive effort combined the energetic demand of metabolism at warmer temperatures (Bayne, 1973) may contribute to the low CI recorded. This may have implications for the farming of *O. angasi* in the northern estuaries in NSW with oysters being more difficult to condition for sale.

Histological examination of *O. angasi* from Port Philip Bay, Victoria, indicated that *O. angasi* were spent during summer and early autumn (Hickman and O'Meley, 1988a). Spent oysters were found in most of the 12 months sampled across all estuaries examined, this may due to the high number of spawning events postulated. In the three northern estuaries sampled, there was an association between number of spent and brooding oysters, while in Merimbula no such association was detected. A rapid change in sex can occur after spawning (O'Sullivan, 1980) and this may have masked a relationship between numbers of spent and brooding oysters in this estuary. All brooding oysters were histologically determined to be male or non-defined and some brooding oysters were assessed as ripe males indicating the quickness of this transition from one sex to another. In contrast, in South Australia 33% of brooding *O. angasi* were female, whether these were residual or developing oocytes was not

determined (O'Sullivan, 1980). Further examination of environmental effects on timing and frequency reproduction between the northern and southern extent of *O. angasi* is warranted as this may give greater insight into the reproductive physiology of this species.

Ostrea angasi is not considered to be true hermaphrodite, but to be sequentially protandric, spawning as one sex then reverting to the alternate sex. It is not known if hermaphrodite *O. angasi* can contribute directly to spawning events as recorded for other *Ostreinae* (Jeffs, 1998), or if these are transitional stage in the protandric sequence of alternating sexual phases. The frequency of hermaphrodites for the 12 months sampled varied among estuaries, 10-20% for the sampling period, and differences between months could be as great as 50% of the population. However, no consistent association was found between the number of hermaphrodites and the number of developing or ripe male and female, brooding, spent or non-defined oysters across the four estuaries. Whether hermaphrodite oysters can spawn or were a transitional phase between males or females was not determined and the contribution to reproductive activity of this species remains unknown.

The temporal patterns of gametogenesis and spawning of *O. angasi* farmed populations in NSW was not consistent with that reported from the southern states, brooding oysters were found for most of the year in NSW and with no defined seasonal reproductive peak. Gametogenesis and spawning of different populations of *C. virginica* have genetically different environmental requirements (Barber et al., 1991), though for other bivalves, such as *C. gigas* (Chavez-Villalba et al., 2003) and *Argopecten ventricosus* (Cruz et al., 2000) timing of gametogenesis is environmentally dependent. Little is known about the relative importance of environment and genetics in determination of gametogenesis in *O. angasi* and the potential differences in gametogenic timing between sub populations in Australia. All oysters sampled were hatchery produced in 2003 from wild stocks sourced from the south coast of NSW. Given the low genetic diversity of the *O. angasi* populations in NSW (Hurwood et al., 2005) and that no planned broodstock selection occurred, broodstock origin is thought to be unlikely to affect gametogenic timing in sub population in NSW.

No consistent association among the four estuaries between proportion of oyster's gametogenic phases and temperature, salinity, CI or brooding oysters, there was. The hottest and coolest water temperatures experienced in these estuaries did not prevent reproductive activity and spawning was asynchronous. *Ostrea edulis* were found to be predominantly

female at the start of the reproductive season following the dormant winter period (Joyce et al., 2013). Further investigation to determine the temperature extremes that constrain spawning of *O. angasi* and the effect this may have on sex ratios and synchronicity of gamete development would assist in hatchery conditioning of brood-stock for larval production and the development of a breeding program. Greater frequency of recording water temperature and salinity to cover tidal cycles may provide a better understanding their effects on *O. angasi* reproductive activity. As hermaphrodites can comprise a considerable proportion of the *O. angasi* population in NSW, further investigation is warranted to determine their contribution to the reproductive ecology of this species.

Chapter 3: The use of neuroactive compounds to chemically induce metamorphosis of hatchery reared flat oyster, *Ostrea angasi*, larvae

3.1 Introduction

Settlement and subsequent metamorphosis of bivalve larvae involves major physiological and morphological changes, including loss of the velum, development of the gill and feeding apparatus, rearrangement of internal organs and secretion of dissoconch shell (Bayne, 1971). These changes indicate the transition from the free-swimming larval phase to the sedentary adult phase. During this period of structural reorganization and morphological change, high rates of mortality occur in both wild and hatchery produced mollusc larvae (Thorson, 1966; Walne, 1974; Rodstrom, 1989; Hann, 1989; Rodriguez et al., 1990; Haws et al., 1993). Inducing rapid metamorphosis of competent larvae can reduce production costs associated with hatchery rearing by producing the desired number of spat and reducing time larvae and spat are retained in the hatchery.

For larvae to metamorphose they must develop the necessary neural network and associated receptors before metamorphosis can occur (Hadfield, 1980; Coon and Bonar, 1987; Degnan and Morse, 1995). The development of the neural network is considered crucial in interfacing larvae with the surrounding environment via chemo and/or mechano-sensors (Burke, 1983) and coordinating metamorphosis (Mesias-Gansbiller et al., 2013). The molluscan larval neural network is complex, comprised an apical sensory organ and paired cerebral, pleural pedal, and visceral ganglia (Ellis and Kempf, 2011). Development of the bivalve larval network initiates in the apical extreme of the trochophore with the appearance of FMRFamide (a neuropeptide having Phe-Met-Arg-Phe-NH_2 sequence) and serotonin immunoreactive cells, both of which contribute to the development of the apical/cerebral complex (Voronezhskaya et al., 2008). Fibres of FMRFamide immunoreactive cells grow posteriorly from this region to innervate the pedal and visceral ganglion. By the pediveliger phase FMRFamide immunoreactive cells are found in the cerebral, pedal and visceral ganglion. The position of the serotonin-like cells do not change during development; however, by the pediveliger phase fibres from these cells extend to innervate frontal and ventral regions including the velum and pedal and visceral ganglion. Concurrent with the development of the FMRFamide- and serotonin immunoreactive cells the distribution and number of catecholamine containing cells have increased in the apical sensory

organ, velum, mantle, oesophagus and large numbers have been detected in the foot (Croll et al., 1997).

The apical sensory organ, given the cells typical sensory cell like structure and that there cilia extend through the epidermal layer, is thought to play an important chemo and mechanosensory role (Bonar, 1978) and has been implicated in the transduction of the metamorphic signal (Croll and Dickson, 2004). Additionally, the neural nature of the apical sensory organ has more recently been accepted (Croll, 2009). By the pediveliger phase the foot is a complex structure, enervated from the cerebral and pedal ganglia (Ellis and Kempf, 2011). The numbers of catecholaminergic cells in the foot increase in the pediveliger phase (Voronezhskaya et al., 2008) and are connected to the neural network by fibre pathways (Croll et al., 1997). Additionally, as the sole of the foot has cilia arising from trans-epithelial apical processes from the catecholaminergic cells and similar cells are were thought to function in a chemo and mechano-sensory role (Bonar, 1978). The maturation of the catecholaminergic pathway in the foot has been considered essential in attaining metamorphic competency (Croll et al., 1997).

Induced metamorphosis in competent larvae is rapid with major morphological changes occurring within 24h (Coon and Bonar, 1987). A variety of chemicals are used to induce larval metamorphosis in mollusc hatcheries. Since the early 1980s metamorphic inducing chemicals including L-DOPA (Cooper, 1983; Coon et al.,1985; Bonar et al., 1990), GABA (Bonar et al., 1990), epinephrine (Coon and Bonar, 1987; Shpigel et al., 1989; Bonar et al., 1990; Garcia-Lavandeira et al., 2005) and epinephrine bitartrate (O'Connor et al., 2008) have been demonstrated to induce synchronous metamorphosis of bivalve larvae. However, not all competent bivalve larvae respond to metamorphosis inducing substances in the same way (Pawlik, 1990; Garcia-Lavandeira et al., 2005): a chemical that induces metamorphosis in larvae of one bivalve species may have no effect or inhibit metamorphosis in other species larvae. Likewise, the concentration and time of exposure to the neuroactive catecholamines required to elicit a response is species-specific (Garcia-Lavandeira et al., 2005).

To maximise the percentage of individuals that undergo induced metamorphosis and survive it is necessary to optimise the combination of chemical, concentration, and period of exposure. Larvae must attain competency, when the necessary receptors for morphogenic induction have developed (Coon and Bonar, 1987; Hadfield, 1980; Degnan and Morse, 1995)

before metamorphosis can occur. Given the non-synchronous development of larvae within a cohort, not all individuals will be physiologically capable of responding to an inducer simultaneously. As a result, hatcheries repeatedly expose larvae to "the inducer chemical" over a series of days, until all individuals that become competent in a cohort have undergone metamorphosis. Decreased rates of metamorphosis have been reported for some molluscan species if pre-competent larvae are exposed to metamorphic inducers (Hadfield, 1980; Avila et al., 1996). Therefore in determining the optimal treatment conditions it is also important to assess the effect of repeated treatment with morphogenic concentrations of catecholamines on the larvae or spat.

Flat oysters, *O. angasi*, have been the basis of a wild harvest fishery for more than a century in several Australian states (Hodson, 1963; O'Sullivan, 1980; Dix, 1980; Hickman and O'Meley, 1988a and 1988b). One factor hampering the hatchery production of flat oyster is the availability of seed and the development of culchless settlement techniques. Initially, hatchery production techniques set oysters spat onto culch (e.g. oyster shells, plastic slats or glass plates) and then either the spat were manually removed for culture or cultured *in situ* (Walne, 1974; Dix, 1980). This technique resulted in high mortalities, so an alternative culch in the form of finely ground and graded (250µm particle size) oyster or scallop shell was used as a settlement substrate. There are, however, limitations in the use of shell culch as a settlement substrate for oyster larvae; pathogens are introduced on the culch, shell dust can affect survival rates and the entrapment of uneaten food and faecal deposition can lead to bacterial proliferation and disease. The use of culch can complicate the spat grading process (size separation and counting of spat) and restrict the use of alternative spat rearing technologies such as spat "bubblers" or fluidisers used rearing Pacific oyster spat. Spat bubblers are a flow through system that maintains the spat in constant suspension in the water column while introducing food in the incoming water and removing waste materials. The production of culchless spat not only provides a solution to these problems, but can reduce labour costs, space (more larvae can be stocked per screen) and the duration of larval cultivation by early induction of metamorphosis.

This study aimed to determine which of the four catecholamines trialed, epinephrine, epinephrine bitartrate, L-3, 4-Dihydroxyphenylalanie (L-Dopa) or γ-aminobutyric acid (GABA), could be used to induce settlement in competent *O. angasi* larvae, ascertain

appropriate dose rates and durations of exposure to the catecholamines and investigate the impacts of prolonged and repeated exposure to catecholamines on metamorphosis.

3.2 Materials and methods

3.2.1 Larval rearing protocols

Flat oysters brooding larvae were obtained from wild stocks on the south coast of NSW (Narooma, Bermagui or Pambula Lake) and opened so larvae could be rinsed into 1L plastics beakers. Larvae were counted and the stage of larval development determined, (i.e. egg to trochophore, D veliger to umbonate larvae, and umbonate larvae to later developmental stages). Larvae were reared using standard protocols employed at the NSW DPI Port Stephens Fisheries Institute (O'Connor et al., 2007). Standard larval rearing conditions of: 1µm filtered sea water at 25 ± 1^{o}C, a salinity of 35 at a stocking density of 2 larvae mL^{-1}. Larvae were fed twice daily and a 100% water exchange every second day.

A total of four experiments were conducted using larvae from at least three different females, except for experiment 3.2.5 where a brood from a single female was used. Larvae were cultured until they were competent and ready to metamorphose. The duration of cultivation varied depending on the initial stage of development of larvae obtained. Collected larvae were examined under 400x magnification using a Leica DME light microscope to determine level of competency and suitability for experimentation. Criteria used to determine competency were the presence of the eye spot, shell length (approx. 340µm), a ciliated foot, the development of 4-5 rudimentary gill filaments and exhibition of pre-metamorphic behaviour (crawling and/or extension of foot). Larvae were carefully sieved and rinsed on 236µm screen and placed in 1L of 25^{o}C temperature equilibrated, 1µm filtered seawater. Five 1mL samples from the 1l container of larvae were counted to estimate total number of competent larvae available for distribution to treatments and controls in each experiment.

3.2.2 General experimental protocols

Four catecholamines were tested in these experiments; epinephrine-bitartrate salt (Epi-B) (Sigma-Aldrich E4375), epinephrine (Epi) (Sigma-Aldrich E4250), L-DOPA (Sigma-Aldrich D9628), and GABA (Sigma-Aldrich A2129). All experiments were conducted in clean, 120mL polystyrene containers (Techno Plas S10844-04) using 1µm filtered seawater (35ppt)

at 25°C fresh filtered seawater (FFSW). Catecholamine solutions were made within 10 min of use for each experiment, when 5mL of distilled water was used to dissolve the test compound and then diluted to the required concentration using FFSW. Using the same treatment durations, controls were exposed to FFSW with the equivalent amount of distilled water used to dissolve the catecholamine. In all experiments, other than experiment 4, Epi-B was used in preference to epinephrine as it is more water soluble. In Experiment 4, to induce water solubility epinephrine was acidified with 0.5mL of HCl in 5mL of distilled water before dilution in seawater to the required concentration, and the equivalent amount of HCl and distilled water was also added to the control treatments in this experiment.

For each treatment three replicates of 200 larvae were treated with the 50mL of catecholamine solution and control replicates treated with 50mL of FFSW with the appropriated quantity of either distilled water or HCl added (Epi treatment only). After treatment larvae were rinsed and maintained in FFSW for a further 24h at 25°C. With the exception of 24h Epi-B and Epi treatments where algae were added after 1h treatment, larvae were not fed during the Epi-B or Epi treatment period. Controls were not fed for the equivalent time period. All treatments and controls were fed a mixed algal diet of Tahitian *Isochrysis* aff. *galbana* (T. *Iso*), *Pavlova lutheri* and *Chaetoceros calcitrans* at 50,000 cells mL^{-1} during settlement trials (Laing, 1995). At the end of this period all treatments, including the 24h treatments (i.e. Experiment 2 and 4, larvae were treated with catecholamine solutions for 24h) were fixed in 10% buffered formalin in seawater for later examination.

At the end of each experiment, all treatments and their respective controls the first 100 larvae were examined for percentage survival, percentage culchless spat (ie. recent metamorphosed non-attached larvae) and the total number of spat (adherent to the container)were counted and expressed as a percentage. Larval metamorphosis was determined by examination at 400x and 200x magnification using a Leica DME light microscope for the loss of the velum and the initiation of the production of the dissoconch shell. Larval mortality was determined from the number of empty larval shells or larvae with marked tissue necrosis.

3.2.3 Induction of larval metamorphosis by GABA, L-Dopa and epinephrine bitartrate

Larvae were pooled and reared in a 1 ton polypropylene tank till deemed competent, using the standard larval rearing protocols detailed above. To determine the optimum

concentration and duration of treatment with Epi-B (Epi-B was used in preference to Epi due to the ease formers solubility in water), GABA or L-Dopa to induce metamorphosis, 3 replicates of *O. angasi* larvae were treated with each of the three compounds (plus controls) at one of four concentrations (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} M) for either of three durations of exposure 0.5h, 1h or 2h in FFSW. Controls were treated using the same protocols, but were exposed to FFSW plus the equivalent amount of distilled water used to dissolve the neuroactive compounds. Twenty-four hours after treatment the samples were fixed before examination.

3.2.4 Effect of prolonged exposure to epinephrine bitartrate

Larvae were pooled and reared in a 1 ton polypropylene tank till deemed competent, using the standard larval rearing protocols detailed above. Four replicates of *O. angasi* larvae were treated with a range of concentrations of Epi-B (10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M) for 24h before being fixed. Controls were treated using the same protocol, but were exposed to seawater with the equivalent amount of distilled water used to dissolve the epinephrine bitartrate. At the end of this period all treatments were fixed before examination.

3.2.5 The effect of multiple exposure to epinephrine bitartrate

Black sick larvae were obtained from a single brooding female, stocked at 2 larvae per mL into a total of 54 replicates 120 mL polystyrene containers, 3 replicates for each treatment daily, sequential and control. All larvae were reared using standard larval rearing protocols (listed above). Treatment with 10^{-3} M Epi-B for 1h in FFSW was initiated prior to larvae displaying cues indicative of competency (ie. low number eyed (<40%), small larval shell size (<340μm and no larvae crawling) to investigate whether multiple exposure to Epi-B has any detrimental effect on metamorphosis and survival of *O. angasi* larvae or short-term survival of Epi-B produced spat into The experiment examined the difference in metamorphosis and survival of larvae treated once daily over a six day period with replicates fixed for later examination daily, i.e. larval replicates treated for 1, 2, 3, 4, 5, or six consecutive days (n=3). The potential for day of exposure to Epi-B to confound the experiment was ascertained by additional larval replicates (n=3) exposed to a single dose of Epi-B on each of the six days. Controls (no treatment) were included, with samples (n=3). Larvae were fixed 24h after treatment.

Spat that had six consecutive Epi-B treatments were reared for a further seven days to determine if there were any short term effects of Epi-B treatment on growth or survival. Thirty spat per replicate (n=3) were reared for seven days in 120mL vials in FFSW, water was exchanged daily. Spat were fed to satiation (O'Connor et al., 1992) on a mixed diet of T. *Iso, P. lutheri* and *C. calcitrans* in the ratio of 2:1:1, respectively.

3.2.6 A comparison of settlement induction by epinephrine bitartrate and epinephrine

Larvae were pooled and reared in a 1 ton polypropylene tank till deemed competent, using the standard larval rearing protocols detailed above. To determine the relative efficacy of different forms of epinephrine, replicates (n=3) of 200 *O. angasi* larvae were treated with epinephrine (Epi) at 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M concentration for 0.5, 1, 2, and 24h. For a comparison replicates were treated with Epi-B at the concentration and duration of exposure that induced the highest rate of metamorphosis in Experiment 1, 10^{-3} M Epi-B for 1h and 24h treated replicates were included to examine any detrimental effects of longer term of exposure. After treatment larvae were rinsed and placed in FFSW and maintained for a further 24h before being fixed. Control replicates were treated using the same protocols, but exposed to seawater with the equivalent amount of 1M HCL and distilled water added.

3.2.7 Statistical analysis

All analyses were conducted using SPSS v.11 software (SPSS Inc. 2001). ANOVAs were used to determine if any significant difference existed between treatments. For Exp. 3.2.3 three way ANOVA was used to determine the effect of three different catecholamines (catecholamine), three different durations of exposure to catecholamine (duration), and four concentrations of catecholamine (concentration) on percentage larval metamorphosis and mortality. For Exp. 3.2.4 a one-way ANOVA with was used to determine the effect of 24h exposure of larvae to four different concentrations of Epi B (concentration) on the percentage of spat produced and larval mortalities. For Exp. 3.2.5 a two-way ANOVA was used to compare the effect of sequential and daily treatment with Epi-B (treatment) for six days on percentage of spat produced or larval mortalities. Where significant differences were found, pairwise comparisons among the means were made using Tukeys HSD ($\alpha = 0.05$). In both two-way and three-way ANOVA where significant interaction were detected between independent variables, the independent variables were pooled for *Post Hoc* comparison (Tukeys HSD, $\alpha = 0.05$). Where interactions were detected between independent variables, graphs depict the pooled, interacting variables compared with the remaining variable.

3.3 Results.

3.3.1 Induction of larval metamorphosis by GABA, L-Dopa and epinephrine bitartrate

There was no significant 3-way interaction among the three variables in the analysis for the number of spat produced ($F_{duration*concentration*catecholamine}$ =1.12, df 12,72, P =0.35). The total number of spat produced was a function of interactions between catecholamine and the concentration of catecholamine ($F_{concentration*catecholamine}$ =22.07, df 6,72, P<0.001), an interaction between catecholamine and duration of treatment ($F_{duration*catecholamine}$ =6.16, df 4,72, P<0.001) and an interaction between concentration of catecholamine and duration of treatment ($F_{concentration*duration}$ =3.11, df = 6,72, P=0.009).

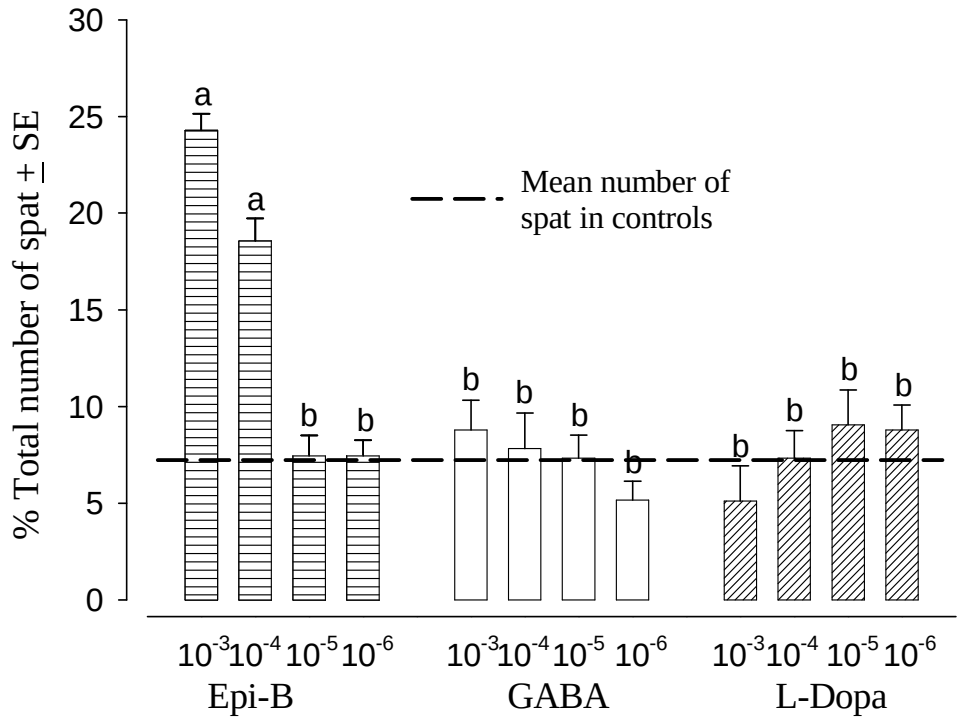

Figure 3.1 Effect of molar dose of catecholamine, Epi-B, GABA and L-Dopa (pooled for duration of treatment) on percent total number of *Ostrea angasi* spat. Means that are not significantly different from one another have the same letter

The average number of spat produced was greatest when larvae where treated with 10^{-3} and 10^{-4} M Epi-B (Fig. 3.1), significantly greater than the controls (3.5 and 2.6 fold greater respectively) and the nearest alternate catecholamine treatment 10^{-5} M L-Dopa (2.5 and 1.9 fold greater respectively). The average number of spat in the remaining treatments did not differ significantly and were consistent with that observed in the controls (mean ± SE, 6.99 ± 1.95).

The greatest average total number of spat produced, was a result of the interaction between treatment duration and concentration of catecholamine (Fig. 3.2), occurred when larvae were treated for 1h at 10^{-3} M concentration of EPI-B. The average number of spat produced from larvae treated for 2h at 10^{-5} and 10^{-6} M concentration of Epi-B was significantly lower (4.8 fold lower for both treatments) than larvae treated for 1h at 10^{-3} M concentration of Epi-B. The remaining treatments did not differ significantly and were similar to the controls.

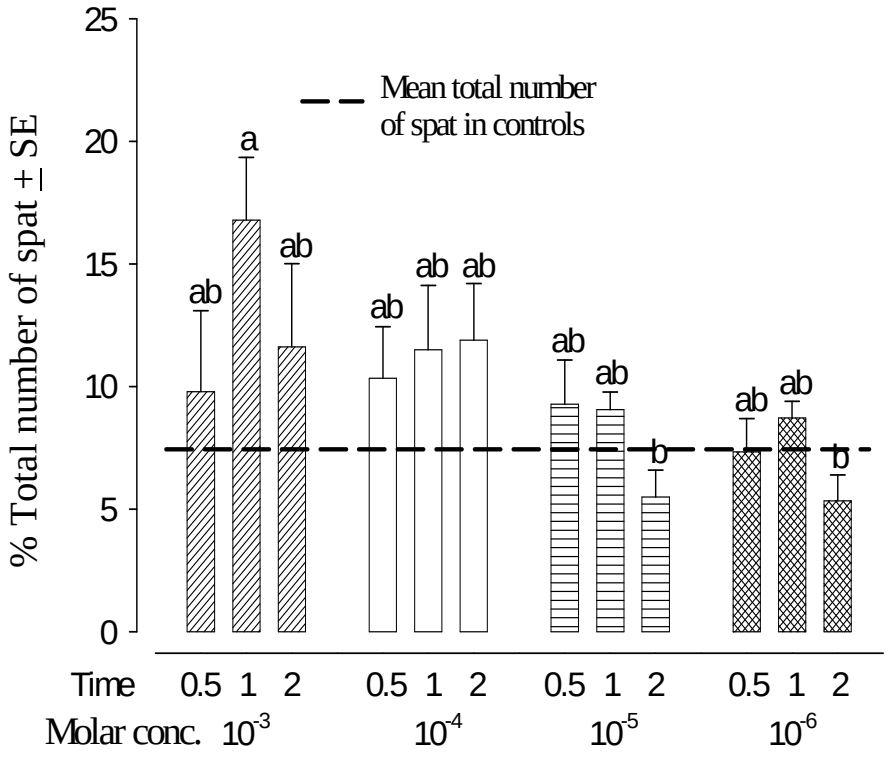

Figure 3.2 Effect of duration of treatment and concentration (M dose) of drug (pooled for catecholamine Epi-B, GABA and L-Dopa) on percent total number of *Ostrea angasi* spat. Means that are not significantly different from one another have the same letter.The greatest

average number of spat produced as the result of the interaction between catecholamine and duration of treatment (Fig. 3.3) was among larvae treated with Epi-B for 1h or 2h. The average number of spat produced among larvae treated with either GABA or L-Dopa for all durations of exposure did not differ significantly. Larvae treated with GABA for 0.5h and L-Dopa for 2h produced significantly less spat (an average 3.1 fold and 3.6 fold lower respectively) than larvae treated with Epi-B for all treatment durations. Larvae treated with GABA for 2h produced significantly less spat (2 fold lower) than larvae treated with Epi-B for 1h and 2h, but did not differ significantly from any of the remaining treatments.

The average number of culchless spat produced was a function of an interaction between catecholamine and concentration of catecholamine ($F_{catecholamine*concentration}$ = 59.23, df 6,72, P<0.001). No interaction was detected between catecholamine and duration of treatment ($F_{duration*catecholamine}$ F=0.589, df 4,72, P=0.156), between catecholamine and concentration of catecholamine ($F_{concentration*duration}$ F=1.613, df 6,72, P=0.156), or among all three variables in the analysis $F_{duration*catecholamine*concentration}$ F= 1.614, df 12,72, P=0.107).

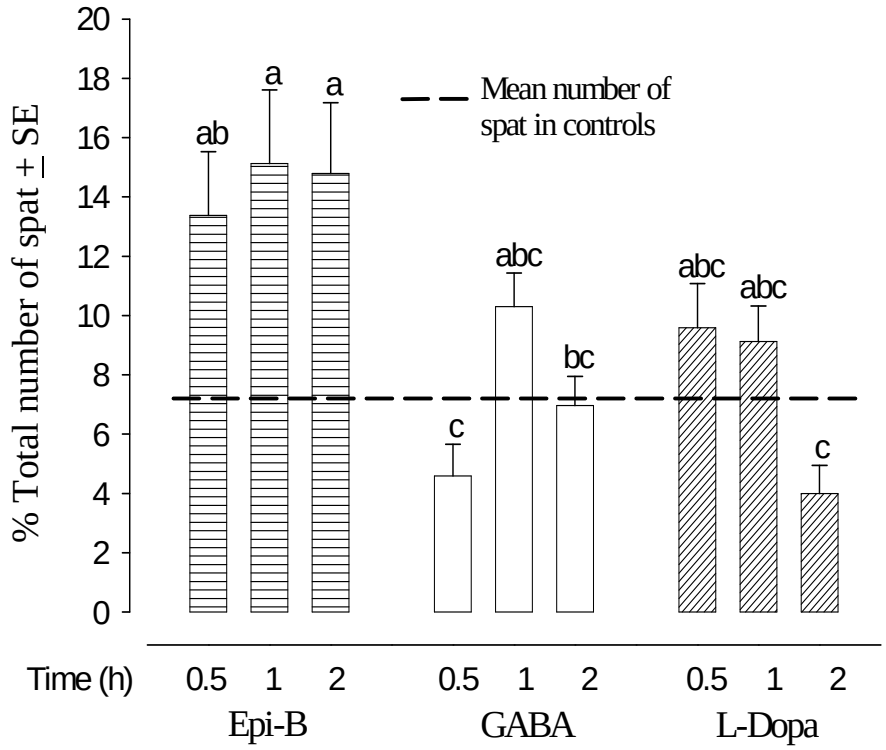

Figure 3.3 Effect of duration of treatment of catecholamine, Epi-B, GABA and L-Dopa (pooled for concentration (M dose) on percent total number of *Ostrea angasi* spat. Means that are not significantly different from one another have the same letter.

The average number of culchless spat produced as a result of the interaction between catecholamine and concentration of catecholamine was significantly greater when larvae were treated with 10^{-3} M Epi-B (a minimum 6.8 fold greater than all other treatments excluding 10^{-4} M Epi-B treatment). The average number of culchless spat produced from larvae treated with 10^{-4} M Epi-B was significantly less (1.4 fold lower) than 10^{-3} M Epi-B treatments, but significantly greater (a minimum 5 fold greater) than all other treatments (Fig. 3.4). The remaining treatments were not significantly different from one another. Duration of exposure (pooled for catecholamine and concentration of catecholamine) affected the average number of culchless spat produced (Fig. 3.5). Larvae treated for 0.5h resulted in a significantly lower number of spat than those larvae treated for 2h, while larvae treated for 1h resulted in spat numbers that did not differ from either 0.5h or 2h.

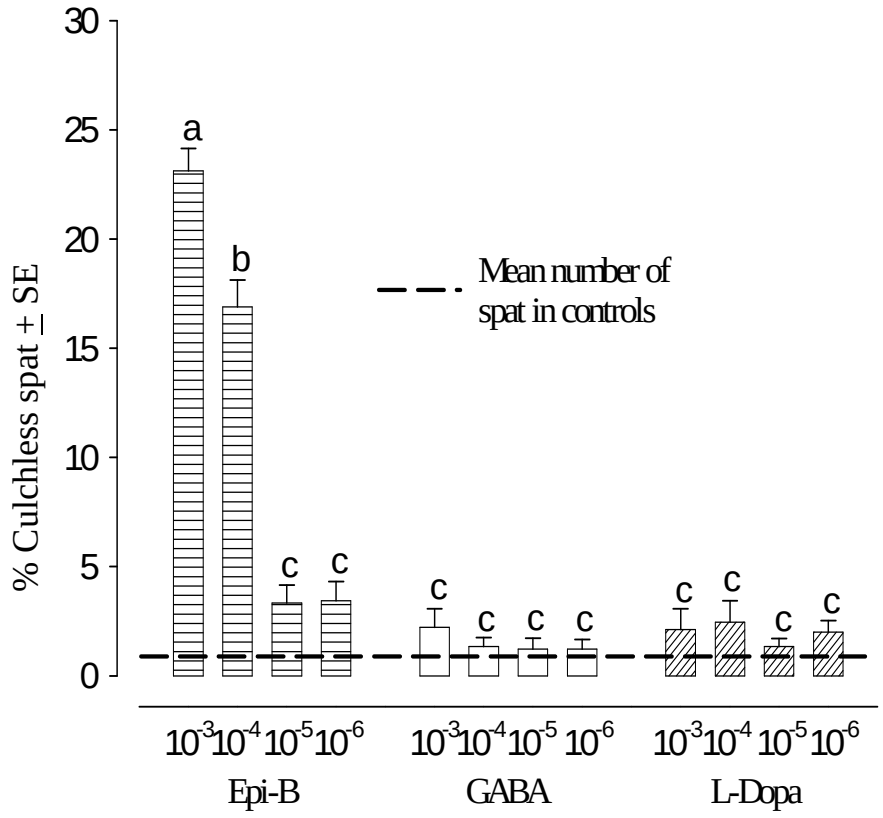

Figure 3.4 Effect of molar dose of catecholamine, Epi-B, GABA and L-Dopa (pooled for duration of treatment) on percent number of culchless *Ostrea angasi* spat. Means that are not significantly different from one another have the same letter.

There was no evidence that any of the three factors (or their interactions) affected the average number of mortalities (mean% ± SE across all samples, 4.4 ± 1.8%), the average number of eyed larvae (69.8 ± 8.0%) or the average size of larvae (346 ± 4µm).

3.3.2 Effect of prolonged exposure to epinephrine bitartrate

Exposure to 10^{-3} M Epi-B for 24h significantly inhibited the percentage of spat produced ($F_{concentration}$=8.972, df 4,15, P<0.001) compared to the controls and the 10^{-5} and 10^{-6} M Epi-B treatments for 24h (Fig. 3.6). There was no difference in percentage mortalities when larvae were exposed for 24h at the different concentrations of Epi-B ($F_{concentration}$=0.516, df 4,15 P=0.725).

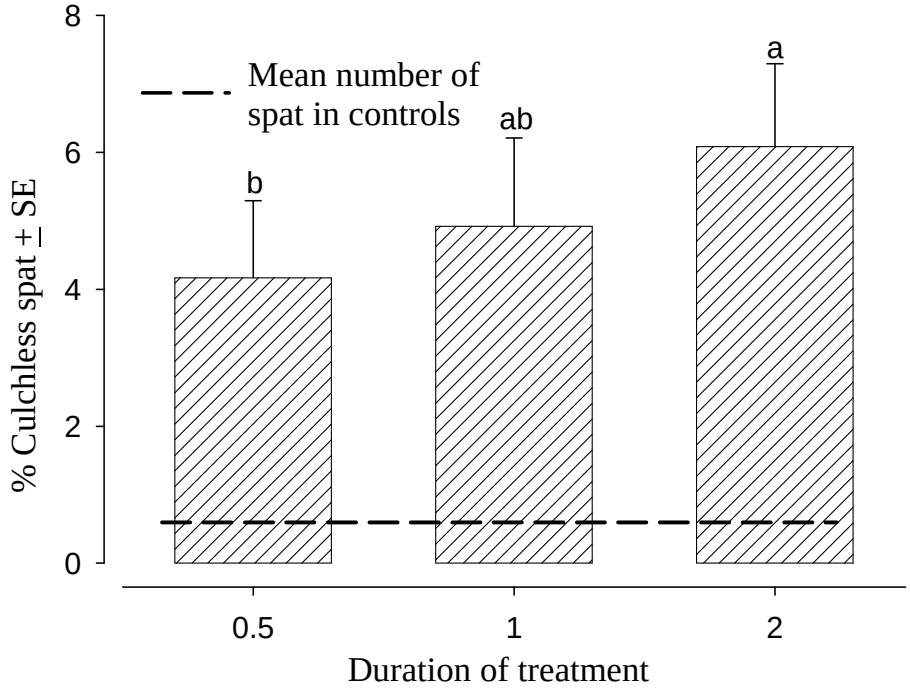

Figure 3.5 Effect of duration of treatment (pooled for M dose and catecholamine) on percent number of culchless *Ostrea angasi* spat. Means that are not significantly different from one another have the same letter.

3.3.3 The effect of multiple exposure to epinephrine bitartrate (Epi-B)

The total number of spat and culchless spat produced was a function of interaction between treatment and day of treatment ($F_{treatment*day}$=12.367, df 10,36 P<0.001). Larvae treated consecutively with Epi-B had higher cumulative total numbers of spat and culchless spat than

larvae exposed to a single daily dose or the controls (Fig. 3.7, A & B). On Days 1 and 2, the total number of spat produced by each treatment did not differ. By Day 3 there were significantly more spat produced from consecutively treated larvae, and by Days 4 and 5 the number of spat produced by both single and consecutive treatments exceeded that of the controls. On Day 6 the larvae treated consecutively produced a significantly greater number of spat than the larvae that received a single treatment, with the latter producing significantly greater number of spat than the controls. The day on which the greatest single daily increase in the total number of spat produced varied depending on treatment. For consecutively treated larvae the greatest single daily increase in metamorphosis occurred on Day 3, averaging 4.2 fold greater than the previous day and 11.7 fold greater than the Day 3 controls. For the single exposed treatments the greatest single daily increase occurred on Day 4, averaging 2.5 fold greater than the previous day and 2.9 fold greater than the Day 4 controls. For the controls the greatest single daily increase occurred on Day 6, averaging a 2.1 fold increase over the previous days control treatment.

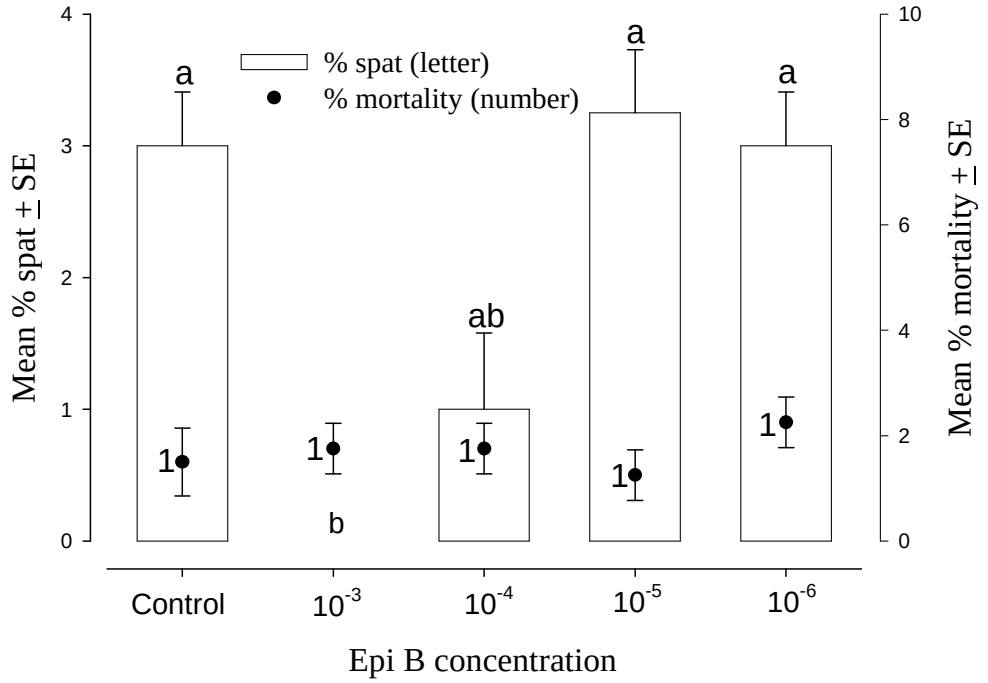

Figure 3.6 Effect of 24 h treatment of *Ostrea angasi* larvae with 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M Epi-B on the mean percentage spat produced and percentage mortalities. Means that are not significantly different from one another have either the same letter or number.

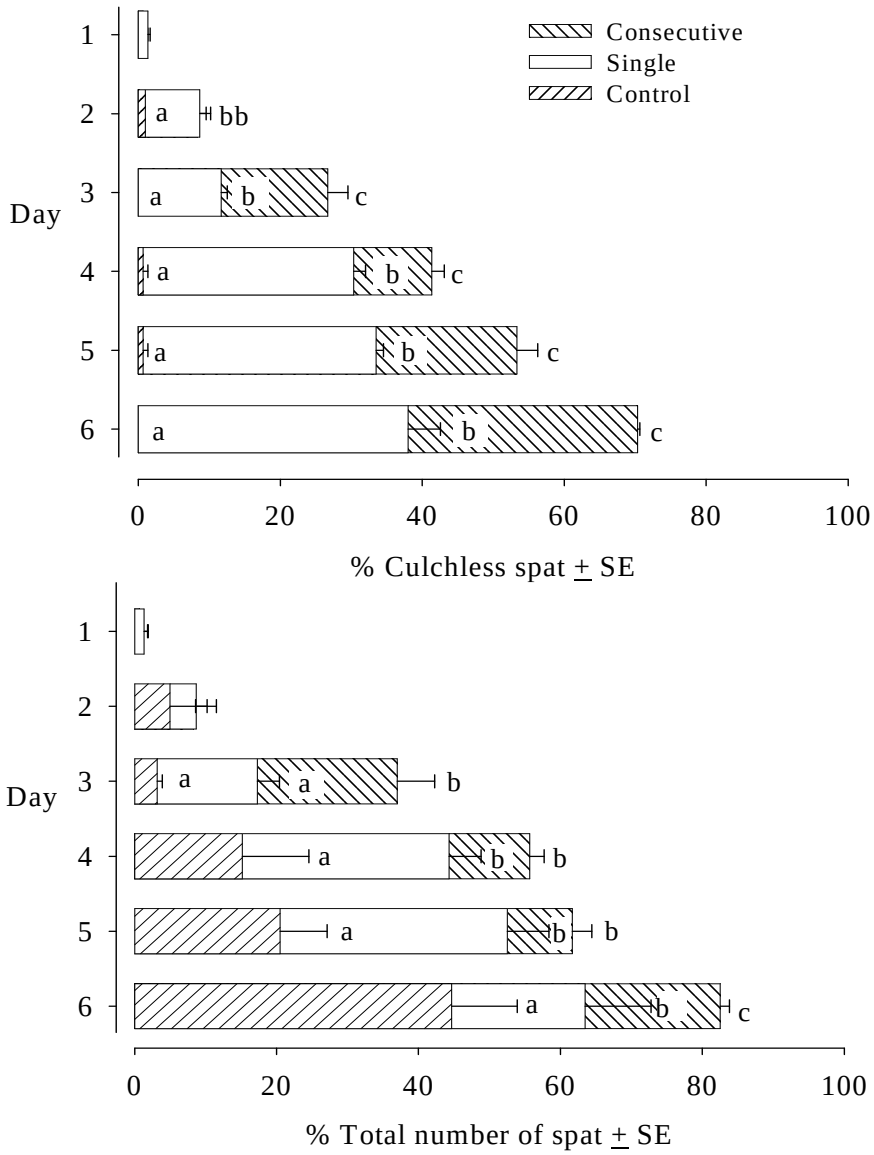

Figure 3.7 Mean percentage cumulative **A**. culchless spat and **B**. total number of spat (pooled for treatment and day) from *Ostrea angasi* larvae treated with 10^{-3} Epi-B for a single dose and consecutively dosed for up to 6 days and no dose (controls). Within day means that are not significantly different from one another have the same letter.

Treatments did not differ significantly in the numbers of culchless spat produced on Day 1. On Day 2, the single and consecutive treatments did not differ but both treatments produced significantly greater numbers of culchless spat than the controls. From then on the consecutively treated larvae had significantly greater numbers of culchless spat than the controls or larvae receiving a single treatment, with the latter treatment producing significantly greater numbers of culchless spat than the controls over the same period.

Neither, treatment or day of treatment had a significant effect on larval mortalities ($F_{treatment}$=0.913, df 2,36 P=0.903 and F_{day}=1.503, df 5,36 P=0.213) and no interaction between the two variable was detected ($F_{treatment*day}$=0.168, df 10, 36 P=0.998).

Spat retained from this experiment and concurrent culch set spat had similar levels of mortality ($F_{treatment}$=0.2, df 1,4 P=0.687) (mean% ± SE, Culch: 1.8 ± 0.3% and 1 ± 0.6%) and shell length ($F_{treatment}$=0.1, df 1,4 P=0.374) (Culch: 518 ± 14.4µm and Epi: 521.7 ± 17.91µm) one week post set.

3.3.4 A comparison of settlement induction between epinephrine bitartrate and epinephrine

Epinephrine was as effective as Epi-B in inducing larval metamorphosis, depending on exposure time, from concentrations of 10^{-3} to 10^{-5}M. However, the highest concentration of Epi used (10^{-3}) had greater mortalities than all other treatments. The percentage of spat produced was consistent when larvae were treated with either10^{-3} molar Epi-B or 10^{-3}, 10^{-4} Epi for all exposure times and 10^{-5} epi for an exposure times of 1 and 2h did not differ in the percentage of spat produced (Fig. 3.8). Treatment with both Epi and Epi-B had a significant effect on the total percentage of spat ($F_{treatment}$=5.87, df 15,32, P<0.001) and culchless spat ($F_{treatment}$=11.95, df 15,32, P<0.001) produced. The treatments that produced significantly greater numbers of culchless spat than the controls were 10^{-3} M Epi-B for 1h, 10^{-4} M Epi for 1h and 10^{-3} M Epi for 2h.

Treatment with 10^{-3} M Epi for 1 and 2h resulted in the highest number of mortalities (mean% ± SE, 20.3 ± 0.9% and 18.7 ± 0.7%, respectively). For short-term treatments (0.5-2h) mortalities differed significantly among the different concentrations of Epi, Epi-B and the controls ($F_{treatment}$=2.08, df 15,32, P=0.04). Though the *Post Hoc* test lacked sufficient power to detect where these differences occurred, it can be concluded that the treatment having the greatest mortality (10^{-3} M Epi for 1h) was significantly greater than the treatment having the lowest mortality (0.5h control larvae).

Treatment for extended time periods (24h) to morphogenic concentrations of Epi and Epi-B exhibited similar, negative effects on metamorphosis as experienced in Experiment 2 (Fig.

3.6). Treatment with 10^{-3} M Epi-B and 10^{-3} and 10^{-4} M Epi for 24h significantly inhibited metamorphosis ($F_{treatment\ 24h}$=35.07, df 5,12, P<0.01). The lower concentrations of Epi did not have the same inhibitory effect. Exposure of larvae for 24h to 10^{-3} M Epi significantly increased the number of mortalities (mean% ± SE, 38.7 ± 5.3%) ($F_{treatment\ 24h}$=14.98, df 5,12, P<0.001), the remaining 24h treatments not varying significantly in the number of mortalities (range 14.3 ± 1.2% to 15± 1.7%).

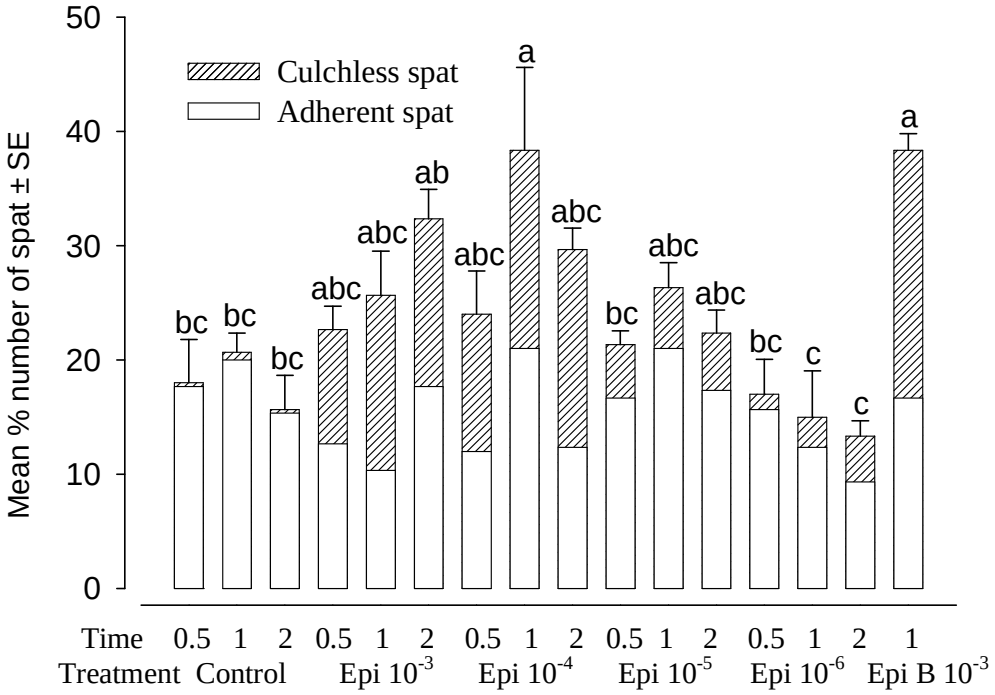

Fig 3.8 Mean percentage *Ostrea angasi* larvae induced to metamorphose in response to increasing concentration of epinephrine treated for 0.5, 1, 1.5 and 2h and controls compared to 10^{-3} M Epi-B exposed for 1h. Means that are not significantly different from one another have the same letter.

3.5 Discussion.

This study has demonstrated that metamorphosis in competent *O. angasi* larvae is affected by catecholamines, the level of response is catecholamine specific and dependent on the concentration and duration of treatment. Furthermore, it reinforces the need for species

specific assessment as these findings for *O. angasi* differ from those results for other closely related species, such as *O. edulis* (Shpigel et al., 1989; Garcia-Lavandeira et al., 2005). Of the catecholamines tested, 10^{-3} and 10^{-4} M Epi-B or 10^{-3}, 10^{-4} and 10^{-5} M Epi for 1 to 2h produced the greatest total number of spat and culchless spat 24h after treatment. The optimum concentration of epinephrine to induce metamorphosis in *O. angasi* larvae (10^{-4} M) was consistent with that reported for other bivalve larvae: *Crassostrea gigas* (10^{-4} M: Coon et al., 1985; Haws and DiMichele, 1993), *Crassostrea virginica* (10^{-4} M: Coon et al., 1986), but is higher than reported for *O. edulis* (10^{-5} M), *Venerupis pullastra*, (10^{-5} M), *Mytilus galloprovincialis* (10^{-5} M) and *Ruditapes philippinarum*, (10^{-5} M) (Garcia-lavandeira et al., 2005). Treatment of both *C. gigas* (Coon et al., 1985) and *C. virginica* (Coon et al., 1986) with 10^{-3} M Epi did not induce any increased metamorphosis above that of the controls. Though treatment of *O. angasi* larvae with 10^{-3} M Epi increased larval metamorphosis the number of mortalities also increased, however treatment with Epi-B (10^{-3}M) and Epi (10^{-3} for 0.5h 10^{-4} and 10^{-5}M for all duration of exposure and 10^{-6}M for 0.5h) produced comparable levels of metamorphosis without an increase in larval mortality. This would indicate the higher concentration of Epi used for longer term exposure is having a toxic effect on larvae. The relative morphogenic efficacy of Epi was higher than that of Epi-B given the lower concentration required to induce larval metamorphosis.

Among the remaining catecholamines assessed, L-dopa and GABA did not increase total number of metamorphosed larvae. Though treatment of *O. edulis* larvae with GABA at 10^{-4} and 10^{-5} M concentration for 48h increased the number of spat produced (Garcia-Lavandeira et al., 2005) it is not clear if the spat were adherent or culchless. The lack of significant response of *O. angasi* larvae to GABA and L-Dopa may be due to the lack of a suitable settlement substrate. When *C. gigas* larvae were treated with L-Dopa and were prevented from contacting the substratum no metamorphosis occurred and 75% of L-Dopa-induced larvae metamorphosing in polystyrene culture vessels ("an unattractive substratum") remained non-cemented (Bonar et al., 1990). Exposure of competent *O. edulis* larvae to Epi for a range of treatment times (45min to 48h) did not affect the number of spat produced, however treatment with 100µM Epi in the presence of a desirable settlement substrate (conditioned shell chips) led to a significant increase in number of spat produced (Shpigel et al., 1989). Exposure of *O. angasi* larvae to catecholamines, GABA or L-Dopa in the

presence of an attractive settlement substrate may induce a greater larval metamorphosis than that observed in this experiment.

Species	Inducer	reference
Haliotis refuscen	GABBA	Morse etal., 1984
Ostrea edulis	GABBA	Mesias-Gansbiller et al., 2013
Ostrea edulis	Epinephrine + substrate	Shpigel et al., 1989
Argopecten purpuratus	epinephrine	Martinez et al., 1999
Crassotrea virginica	epinephrine	Coon et al., 1986
Ruditapes philippinarum	epinephrine	Lu et al., 2006; Garcia-lavandiera et al., 2005
Crassotrea belcheri	epinephrine norepinephrine GABBA L-Dopa	Tan and Wong, 1995
Crassostrea gigas	epinephrine	Coon et al., 1986
Pecten maximus	Epinephrine L-Dpoa	Chevelot et al., 1991

Table 3.1 Catecholamines used to successfully induce larval metamorphosis in mollusc larvae

For induced larval metamorphosis the development of the necessary neural network and associated receptors is essential (Coon and Bonar, 1987). The mechanism of action of metamorphic inducing substances and the location of the receptors sites for the majority of different bivalve larval species remains unknown. The requirement of some bivalve larvae for a settlement substrate in the presence of a metamorphic inducer to increase larval metamorphosis may indicate a different mechanism of action on the metamorphic pathway. Some catecholamines have been indicated to operate at different site within the metamorphic pathway, L-Dopa eliciting a settlement response but without a suitable substrate present the irreversible step of metamorphosis does not occur (Coon et al., 1990). As with *O. angasi*, epinephrine has been indicated to bypass the L-Dopa driven response and activate the metamorphic process directly (Bonar et al., 1990; Garcia-Lavandeira et al., 2005). Surface topography has also been reported to induce high levels of metamorphosis in *P. canalicucla* (Gribben et al., 2011) and the addition of a metamorphic inducer as well as settlement substrate has been shown to increase larval metamorphosis (Shpigel et al., 1989), possible as a result of activation of different mechanisms within the metamorphic pathway (Coon et al., 1990). Whether GABA, L-Dopa and epinephrine are acting on different location within the "competence-metamorphic" pathways has not been determined and further research into the mechanism of action is warranted.

Prolonged treatment (24h) of *O. angasi* larvae to Epi-B at 10^{-3} M and Epi at 10^{-4} M inhibited larval metamorphosis without increased mortality. Prolonged treatment (24h) of *O. angasi* larvae to 10^{-3} M Epi more than doubled the number of mortalities and increased level of mortality were observed in the short-term 10^{-3} M Epi treatments compared with all other treatments. Therefore this concentration of Epi is not recommended as a treatment for spat production. Increased mortalities occurred when *Pinctada margaritifera* larvae were treated with 10^{-3} M GABA for prolonged periods (Doroudi and Southgate, 2002). Treatment of *C. gigas* larvae for 40 to 60 min with 10^{-4} M Epi induced maximum larval metamorphosis, with no decrease in the number of metamorphosed larvae after prolonged treatment (i.e. 24h) (Coon and Bonar, 1985; Coon et al., 1986). *O. edulis* larvae treated with 100 µM Epi alone (i.e. no settlement substrate), for 45min to 24h and 48h did not affect the number of spat produced (Shpigel et al., 1989). In contrast continuous treatment of *O. edulis* larvae with 10^{-5} M Epi for 48h, (24h data not presented) increased the number of spat produced (Garcia-Lavandeira et al., 2005).

Non-lethal inhibition of metamorphosis may be of use in examination of the biochemical and physical events surrounding this transitional phase. Inhibition of metamorphosis with prolonged treatment with Epi was noted for *Crassostrea belcheri* (Tan and Wong, 1995) though the authors also noted increased levels of larval mortalities. The metamorphic inhibitory effect of prolonged treatment of *O. angasi* larvae with morphogenic concentrations of Epi-B or Epi is reversible after transfer to FFSW for 24h. The ability to synchronise settlement and metamorphosis of *O. angasi* larvae using of the reversible inhibitory effect would be useful in hatchery production of this species. However inhibition of metamorphosis of *O. angasi* was not successful at low concentrations and cost of chemicals would preclude the use of this method to attempt to inhibit metamorphosis in competent larvae for commercial production.

Consecutive exposure of *O. angasi* larvae to morphogenic concentrations of Epi significantly increased the number of culchless spat with no increased mortalities of larvae or spat (1 week post-treatment). Epinephrine treated *C. gigas* and *C. virginica* showed no negative effects on development or survival for 12 months post metamorphosis (Coon et al., 1986). Similarly, *O. edulis* larvae did not suffer any deleterious effects on growth or survival for 120 days post set (Shpigel et al., 1989), nor did *O. angasi* spat for 1 week post set. Of interest is the sequence

of increase in spat produced between the three treatments. Larvae treated consecutively with Epi-B exhibited greater numbers of spat produced earlier than the other treatments, with a concomitant reduction in the number of eyed larvae observed in these treatments. Catecholamines have been implicated in a developmental role in *Pecten maximus* larvae (Croll et al., 1997; Robert et al., 1999; Cann-Moisan et al., 2002) and have been shown to effect larval sensitivity at metamorphosis. Additionally, incubation of the aeolid nudibranch, *Phestilla sibogae* larvae with L-Dopa was shown to increase larval sensitivity to their natural metamorphosis inducer (Pires et al., 2000). Consecutive treatment with Epi-B or Epi was found to affect the rate of development of competency this warrants further investigation of role of these catecholamine's in development process of *O. angasi* larvae.

Treatment of competent *O. angasi* larvae to 10^{-4} M Epi or 10^{-3} M Epi-B can be reliably used to induce metamorphosis of *O. angasi* as culchless spat with no short-term detrimental effects. It is interesting to note the differing morphogenic effects of epinephrine on *O. angasi* and *O. edulis* (Shpigel et al., 1989; Garcia-lavandeira et al., 2005) which are believed to be very closely related species (Hurwood et al., 2005). Treatment with Epi does not require the presence of a suitable settlement substrate to induce metamorphosis. The removal of the culch materials (shell grit) offers advantage in easy determination of numbers of metamorphosed and non-metamorphosed larvae during set, greater numbers of spat can be retained per screen once metamorphosed. The ability to reliably and rapidly induce metamorphosis in competent larvae offers a valuable tool to examine the effects of larval cultivation techniques (diet, temperature, etc.) on larval development and acquisition of competency. Non-replicated comparison of Epi-B set and shell set have shown both techniques to be equivalent in the numbers set, the former giving faster results with the previously discussed advantages. Use of the correct protective equipment and handling procedures for use of this catecholamine must be observed and government agency approvals should be obtained. Treatment of *O. angasi* larvae with 10^{-4} M Epi for 1h, when larvae are greater than 60% eyed and are exhibiting crawling behaviour, can decrease the time taken for successful larval settlement and metamorphosis and offer the additional advantages of the removal of culch materials.

For hatchery production of *O. angasi* larvae, consecutive daily treatment with 10^{-4} M Epi or 10^{-3} M Epi-B for 1h when approximately 60 - 80% of larvae are eyed will increase the rate of settlement and metamorphosis and number culchless spat produced. Low induction rates can

result from having a large proportion of larvae in a population not yet competent to respond to treatment with a morphogenic inducer (Shpigel et al., 1989). Using larval shell size to separate larvae into as uniform size range as possible before treatment and determining the proportion of eyed and crawling larvae will aid in treatment success. Additionally, the capability to induce rapid metamorphosis in competent *O. angasi* offers an invaluable tool to assess the effects of rearing conditions on larval development and acquisition of competency.

Chapter 4. Dietary influence on growth and development of flat oyster, *Ostrea angasi* (Sowerby, 1871), larvae

4.1 Introduction

The importance of diet for hatchery rearing of bivalve larvae is well documented (Walne, 1974; Nell and O'Connor, 1991; Aldana-Aranda and Patino Suarez, 1998; Rico-Villa et al., 2006), as are ontogenetic changes in dietary preference or nutritional requirements during larval development in culture (O'Connor et al., 1992; Pernet et al., 2006). Considerable inter-specific variation occurs in bivalve larval dietary requirements (Coutteau and Sorgeloos, 1992). Though the relationship between gross biochemical composition of microalgae and the nutritional value of microalgae is not clear (Jeffery et al., 1994), the composition of each fraction is of greater importance than the gross composition (Helm et al., 1973). Fatty acids, in particular the polyunsaturated fatty acids (PUFA), are considered essential for bivalve larval growth and development (Langdon and Waldock, 1981; Leonardos and Lucas, 2000a & b).

Selection of algal species for bivalve diets has arisen from algal species availability, ease of algal culture, and success with culture of other bivalve larvae. Biochemical analysis of algae can be used to suggest suitable species (Langdon and Waldock, 1981), but larval dietary trials are most commonly used (Webb and Chu, 1982). This latter approach allows observation of ingestion, digestion, nutritional acquisition and subsequent larval growth and development. Generally, diets combining several microalgal species result in increased larval growth rates and development (Walne, 1974; Helm, 1977). In a hatchery context it is important to know which algal species will provide optimum growth, survival and development for a particular larval species.

Metamorphosis is a critical, energy consuming transitional phase of early bivalve life history (Bartlett, 1979; Rodriguez et al., 1990; Laing, 1995) when high mortality combined with variable settlement and metamorphosis are often reported (Garcia-Esquivel et al., 2001; Pernet et al., 2006; Rico-villa et al., 2006). During metamorphosis ingestion of algal cells stops or decreases, corresponding with the loss of the larval velum and subsequent development of the adult gill and digestive system (Holland and Spencer, 1973). Successful

metamorphosis by larvae has been correlated with maternally derived nutrient reserves in the egg (Bayne et al., 1975; Gallager and Mann, 1986; Helm et al., 1991; Berntsson, 1997) and nutrients acquired during the larval phase (Ferreiro et al., 1990; Videla et al., 1998; Jonsson et al., 1999). It is therefore important to assess successful metamorphosis among larval performance indicators when evaluating suitable diets.

Hatchery production of the flat oyster *Ostrea angasi* has occurred in Australia since the early 1970's (Anon, 1989; Dix, 1980; Hickman and O'Meley, 1988b), but has been hampered by high mortalities during larval settlement and metamorphosis. These losses may be related to the quality of the algal diet, yet the effects of different algal diets on growth, survival and metamorphosis of *O. angasi* larvae have not been assessed. The aim of this study was to determine the best hatchery diet for *O. angasi* larvae from among 24 different microalgal diets (unialgal, binary and ternary diets) composed of eight commonly used microalgal species by assessing growth rate, survival, development and metamorphosis of larvae fed each diet.

4.2 Materials and Methods

4.2.1 Larval collection

Flat oyster brood-stock were obtained from wild stocks on the south coast of NSW (Narooma, Bermagui or Pambula Lake). Larvae were removed from narcotised females using MgCl (50 g l^{-1} in 25% sea water/75% freshwater: Butt, et al., 2008) and rinsed thoroughly in 1µm filtered sea water (FSW). Each batch of larvae was counted and stage of larval development recorded. Each experiment used larvae of a similar size and stage of development that were collected from at least three different females.

4.2.2 Larval rearing

In all trials, larvae were reared in a Paton Scientific (Model 013422) orbital shaker incubator maintained at 25 ± 1^{o}C with no agitation. Larvae were cultured in 200mL of 1µm filtered, 35 salinity FSW in 250mL conical flasks at a density of 2 larvae mL^{-1}. Larvae were fed twice daily and a 100% water exchange every second day. Three replicate larval containers were used for each diet in each trial and each trial included three replicate flasks of unfed larvae as negative controls. Two larval stages examined were early stage larvae (ie. branchially retained eggs to larvae approximately 230µm shell length) and late stage larvae

(ie. free swimming larvae approximately 230 to 340µm shell length) (Hickman and O'Meley, 1988b). Embryos were collected and pooled from 4 brooding females. For the early stage dietary trials (Experiment 1) embryos were collected, counted and reared to D-veliger stage of development, approximately 140µm (O'Sullivan, 1980) and the initiation of the early stage of larval development unialgal dietary trail. Larval replicates were cultured for eight days and fed separate unialgal diets until they reached approximately 230µm shell length, the size at which larvae are normally released from the branchial chamber of the adult oyster (Hickman and O'Meley, 1988b) and cessation of early stage larval development dietary assessment. For trials using late-stage larvae (Experiment 2 & 3) additional embryos, the same as used in the early-stage trial, were reared concurrently in a 1000L tank and fed a mixed algal diet comprising an equal mixture of all eight algal species following the protocols detailed above. Larvae were reared to a mean shell length of 224µm before use in the late larval stage development diet unialgal trials. Larvae reared for the assessment of unilagal diet effect on early stage larvae were reared for 8 days only.

In the late stage diet trials, larval competence to metamorphose was assessed by exposing the larval replicates to epinephrine bitartrate (Epi-B) at 10^{-3} molar concentration for 1h (O'Connor et al., 2009). Timing of Epi-B treatment for experimental larval replicates was ascertained from larvae reared concurrently using the same experimental rearing conditions. When concurrently reared larvae exhibited signs of competency to metamorphose experimental larval replicates were treated with Epi-B. Concurrently reared larvae were fed T. Iso in uni-algal diet trials, T. Iso and *T. chuii* in binary diet trials, or T. Iso, *T. chuii* and *N. oculata* in ternary algal diet trials. Competency was inferred by the presence of the eye spot, shell length of approx. 340µm, a ciliated foot, development of 4-5 rudimentary gill filaments and exhibition of pre-metamorphic behaviour (crawling and/or extension of foot). After 1h treatment with Epi-B, larvae were returned to their culture vessels in FSW for a further 24h, with the appropriate experimental algal diet, before fixing with 10% formalin in seawater for measurement and enumeration. The shell length of 30 randomly selected larvae from each replicate was measured to assess the effect of diet on growth rates. The daily specific daily growth rate (SDGR, *k*) was calculated as:

$$k = (\ln SL_1 - \ln SL_0) / t_t$$

where SL_1 is shell length at the end of cultivation time t_t (days) and SL_0 is the shell length at the start of the trial. For all late stage larval trials, the percentage of eyed larvae, mortalities, and spat produced were determined from the first 100 larvae of the total larvae viewed (100x magnification). Larval mortality was estimated from the number of empty larval shells or larvae with marked tissue necrosis and expressed as a percentage.

Microalgae used in the diet trials were selected on the basis of common use in molluscan hatcheries (Coutteau and Sorgeloos, 1992), their past performance in dietary trials on bivalve larvae cultured at the Port Stephens Fisheries Institute, NSW (Nell and O'Connor, 1991), their physical characteristics and biochemical profiles (Table 4.1), and those species used to culture *O. angasi* larvae previously (O'Sullivan, 1980; Dix, 1980; Hickman and O'Meley, 1988a). All microalgae were cultured semi-continuously in 10l polycarbonate vessels (Aquatek, SA), under cool-white fluorescent light exposed to a 16h:8h, light:dark photoperiod at 23^{o}C ± 1^{o}C using f/2 growth media (Guillard, 1975). Algal culture conditions can affect the algal lipid composition (Leonardos and Lucas, 2000a), to minimise potential variation of nutritional profile of algal cultures used (Brown et al., 1997; Jacobsen et al., 2010), a minimum of two and a maximum of five separate cultures of each algal species were harvested and mixed for each dietary trial. The microalgal cell concentrations in the algal cultures were determined every second day by triplicate cell counts using a Neubauer haemocytometer. Larvae were fed each algal species (or mix of species) on an equal dry weight basis (Nell and O'Connor, 1991; Nell et al., 1994), using a dry weight basis feed formula developed for Sydney rock oysters (O'Connor et al., 2008) twice daily. All algal cultures were screened on thiosulphate citrate bile salts sucrose agar plates for contamination with potentially pathogenic *Vibrio* spp. prior to use, any algal cultures suspected of contamination with *Vibrio* were discarded and not used in these trials.

4.2.3 Unialgal dietary trial

Embryos for both larval stages from unialgal dietary assessment were taken from four brooding females pooled and reared until they had reached the D-stage of larval development (with a shell of approximately 140 µm) for early stage larvae and an additional batch of pooled larvae were on reared until a shell length of 224 µm from late stage larval dietary assessment. To determine the best unialgal diet and investigate possible ontogenetic shifts in dietary preference or nutritional requirements, larvae were fed uni-algal diets of each of the eight selected microalgal species during two stages of larval development, as described

below. Algal species used during these dietary trials where *Isochrysis sp.* (T. Iso), *Pavlova lutheri, Nannochloropsis oculata, Tetraselmis chuii, Skeletonema marinoi, Chaetoceros muelleri, Chaetoceros calcitrans* and *Thalassiosira pseudonana.* The two stages examined were early stage larval development (ie. branchially retained embyos) and late stage larval development (ie. free swimming larvae approximately 230 µm shell length) (Hickman and O'Meley, 1988b). For the early stage dietary trials, embryos were collected, counted and cultured to D-veliger stage, approximately 140µm shell length (O'Sullivan, 1980). Larval replicates were cultured for eight days and fed separate unialgal diets until they reached approximately 230µm shell length, the size at which larvae are normally released from the branchial chamber of the adult oyster (Hickman and O'Meley, 1988b). Early stage larvae were not reared further than eight days and were only used in the early stage unialgal diet.

For the unialgal dietary trials using late-stage larvae, additional larvae, the same embryos collected and used in the early-stage trial, were pooled and reared concurrently in a 1000 L rearing vessel and fed a mixed algal diet comprising an equal mixture of all eight algal species following the protocols detailed above. Larvae were reared to a mean shell length of 224µm before use in the late larval stage development single algal diet trials.

4.2.4 Binary algal diet trial

Late grey sick (200µm shell height) were collected and pooled from three brooding females for the binary algal diet trial. Microalgae used in this trial were selected on the basis of their performance over the four parameters measured in the unialgal species trials with late stage *O. angasi* larvae, with emphasis on the percentage of spat produced. The two better unialgal diets (*T. chuii* or T. Iso) were fed in a pair wise combination, and in combination with either *C. calcitrans, C. muelleri* or *P. lutheri* on a 1:1 dry weight basis to late stage *O. angasi* larvae. Single algal diets of T. Iso and *T. chuii* were included as a comparison between single and more complex algal diets.

4.2.5 Ternary algal diet trial

Late grey sick (195µm shell height) larvae were collected and pooled from three brooding females for the ternary algal diet trial. The algal species used in this trial were selected on the basis of the binary diet trials. The best binary diet combination (T. Iso and *T. chuii*) was fed to late stage *O. angasi* larvae, in combination, 1:1:1 on a dry weight basis with a combination of the remaining five algal species, *P. lutheri, C. calcitrans, C. muelleri, N.*

occulata or S. marinoi and T. Iso and *P. lutheri* in combination with *C. calcitrans* or *C. muelleri*. Single algal diets of T. Iso, *T. chuii*, *C. muelleri* and *D. tertiolecta* and a single binary diet of T. Iso and *T. chuii* were included as a comparison between single and more complex algal diets.

4.2.6 Statistical analysis

All statistical analyses were conducted using SPSS software (v.11, SPSS Inc. 2001, Chicago, Illinios, USA). Homogeneity of variance was checked using Cochran's test and residual plots. When necessary, percentage data was arc sin square root transformed to correct for heterogeneity of variance. To examine the effects of the unialgal diets on early and late stage larvae from a combined larval cohort a two ANOVA was used to compare the effects of 7 unialgal diets on 2 stages of larval development (development stage) on growth and survival. Only later stage larvae were reared on the unialgal diet to assess larval development, percentage eyed larvae and spat produced. To examine the effects of unialgal (9 diets), binary (10 diets) and ternary diets (14 diets) on late stage larvae (>190µm shell height) a one-way ANOVA was used to examine dietary effects on larval growth, survival, development to eyed stage of larvae and larval metamorphosis. When significant differences were found, pair-wise comparisons among the means were made using Tukeys HSD test (Sokal and Rolhf, 1981). The relationship between percentage of spat produced and either SDGR or percentage of eyed larvae was examined using linear regression to determine if either could be used as a reliable predictor of larval competency.

4.3 Results

4.3.1 Unialgal diet trial

Both diet and stage of larval development had a significant effect on larval mortality (F_{diet}= 4.10, df 8,36, P=0.001 and $F_{developmental\ stage}$= 6.37, df 1,36, P=0.16, respectively) and no interaction was detected between the two factors ($F_{developmental\ stage*diet}$ =0.92, df 8,36, P=0.51). Late stage larvae had, on average, 1.3 fold greater larval mortality than early stage larvae. When larvae from both stages of development where fed *T. chuii* or *S. marinoi* they averaged 2 fold lower percentage mortalities when compared to larvae fed *C. calcitrans* (Fig. 4.1). The remaining diets, averaging 16.3 ± 2.3 (mean % mortality ± SE), did not differ in mean percentage mortalities.

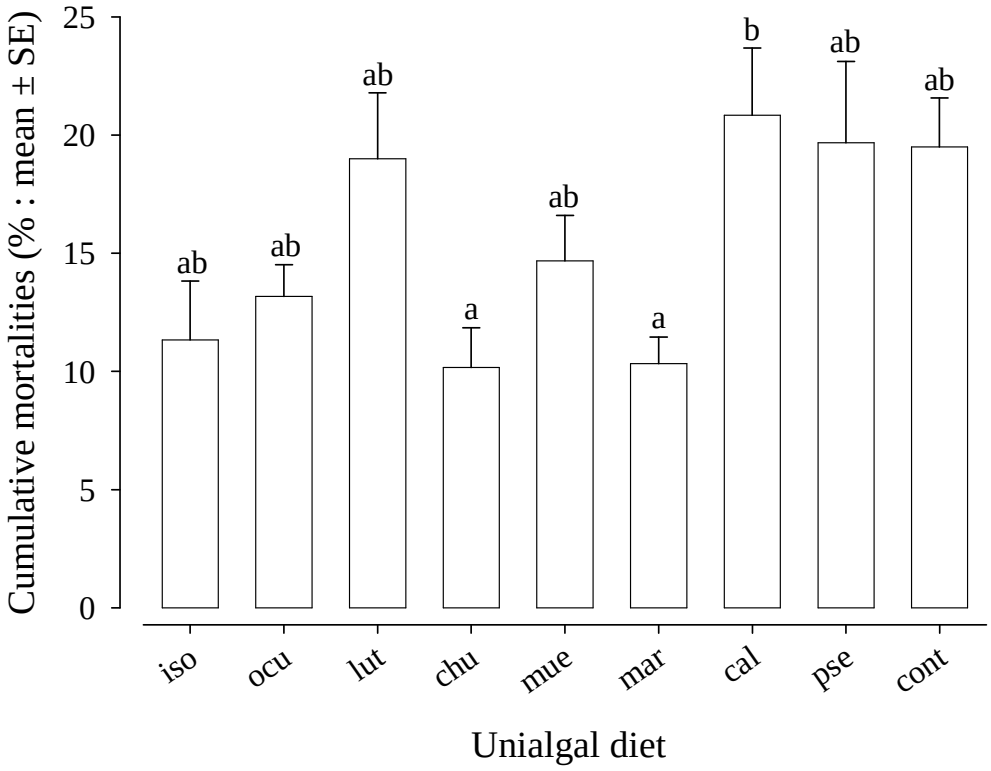

Figure 4.1 Mean cumulative mortalities (%) recorded on day 18, pooled of *Ostrea angasi* larvae fed 8 unialgal diets (*P. lutheri* (lut), *Isochrysis* sp. (Tahitian) (iso), *C. muelleri* (mue), *C. calcitrans* (cal), *S. marinoi* (mar), *T. pseudonana* (pse), *T. chuii* (chu) or *N. oculata* (ocu) and unfed larvae (cont). Means with the same letter are not significantly different from one another.

The average larval growth rates were a function of the interaction between algal diet and stage of larval development ($F_{developmental\ stage*diet}$=4.05, df 8,36, P=0.002). Post hoc comparison of the effect of the unialgal diets on the different stages of larval development indicated the least nutritious unialgal diets had a more pronounced effect on larva growth during the later stage of larval development (Fig. 4.2).

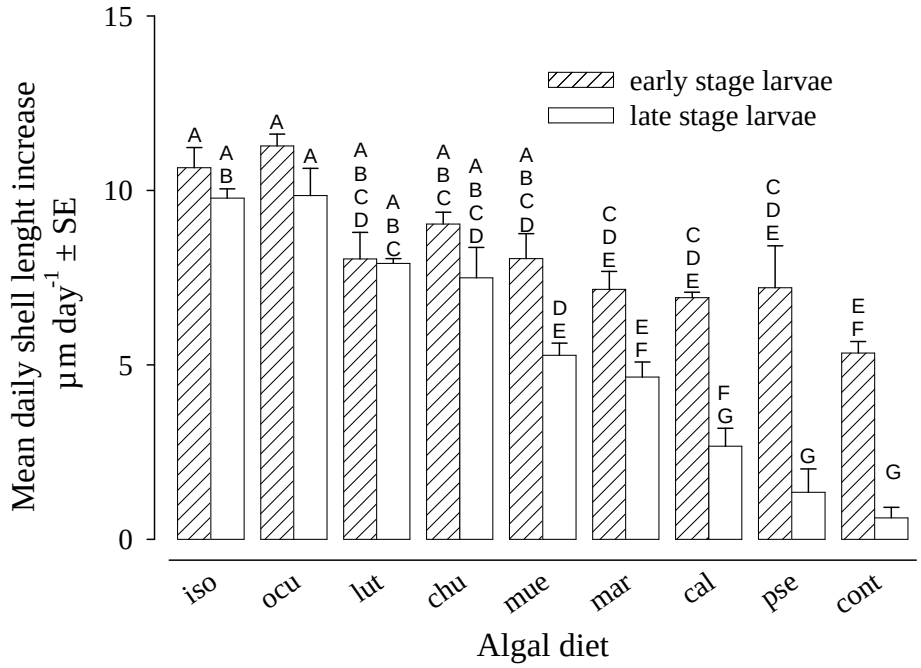

Figure 4.2 Mean daily growth rates for early (day 8) and late (day 18) development stage, *Ostrea angasi* larvae fed unialgal diets (*P. lutheri* (lut), *Isochrysis* sp. (Tahitian) (iso), *C. muelleri* (mue), *C. calcitrans* (cal), *S. marinoi* (mar), *T. pseudonana* (pse), *T. chuii* (chu) or *N. oculata* (ocu) and unfed larvae (cont). Means with the same letter are not significantly different from one another.

A significant difference was detected among diets in mean percentage eyed larvae produced ($F= 36.22$, df 8,18, $P<0.001$) with larvae fed T. Iso, *N. oculata*, *T. chuii* or *P. lutheri* producing the greatest percentage of eyed larvae, averaging 10.2 fold greater percentage of eyed larvae compared to larvae fed the remaining diets or unfed larvae. Larvae fed *T.*

pseudonana or *C. calcitrans* or larvae that were unfed did not develop to the eyed stage (Fig. 4.3).

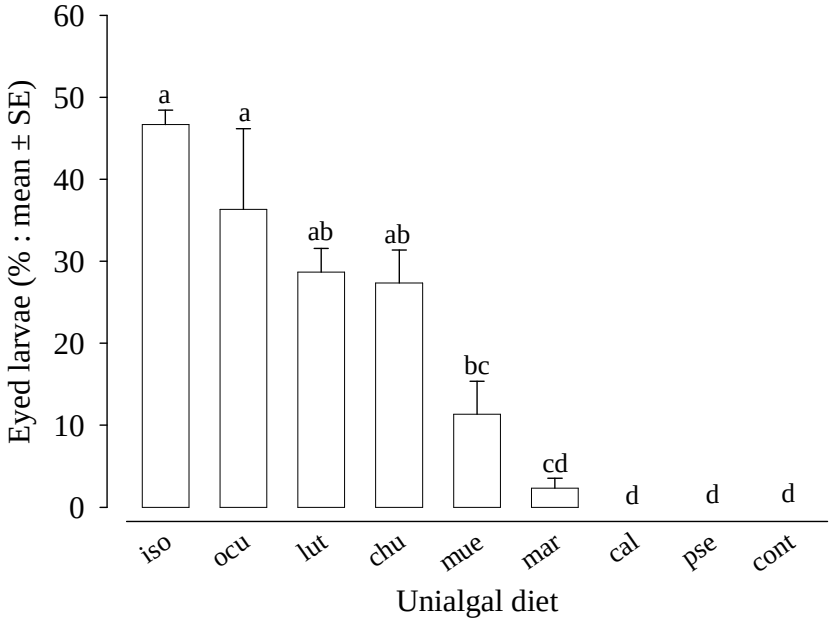

Figure 4.3 Mean *Ostrea angasi* eyed larvae (%) on day 18, previously fed unialgal diets, *P. lutheri* (lut), *Isochrysis* sp. (Tahitian) (iso), *C. muelleri* (mue), *C. calcitrans* (cal), *S. marinoi* (mar), *T. pseudonana* (pse), *T. chuii* (chu) or *N. oculata* (ocu) and unfed larvae (cont)). Means with the same letter are not significantly different from one another.

The mean percentage of spat produced differed among the diets ($F= 13.78$, df 8,18, $P<0.001$). Larvae fed *N. oculata*, T. Iso or *T. chuii* diets yielded the greatest percentage of spat (Fig 4.4). Larvae fed. *C. muelleri, S. marinoi, C. calcitrans, T. pseudonana* failed to develop to competency, as did unfed larvae A significant, positive correlation was detected between percentage eyed larvae and percentage of spat produced ($r=0.82$, $n=27$, $P<0.001$), and between growth rates and percentage of spat produced ($r=0.75$, $n=27$, $P<0.001$).

Based on their positive effects on the performance of late stage *O. angasi* larvae, ease of cultivation, temperature tolerance and cell densities achievable in culture at the Port Stephens

Fisheries Institute, T. Iso and *T. chuii* were used in binary algal diets trials (*N. oculata* was not available for the Binary diet trial).

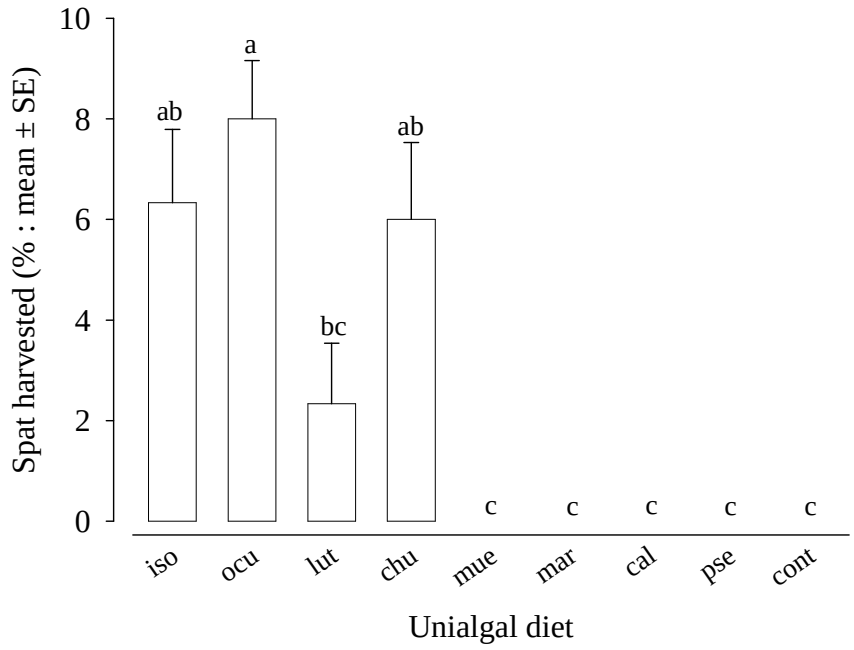

Figure 4.4 Mean *Ostrea angasi* spat (%) harvested day 18, previously fed unialgal diets (*P. lutheri* (lut), *Isochrysis* sp. (Tahitian) (iso), *C. muelleri* (mue), *C. calcitrans* (cal), *S. marinoi* (mar), *T. pseudonana* (pse), *T. chuii* (chu) or *N. oculata* (ocu) and unfed larvae (cont)). Means with the same letter are not significantly different from one another.

4.3.2 Binary algal diet trial

Diet affected larval growth rates ($F=43.05$, df 9,20, $P<0.001$). The slowest growth rates, by a factor of two, were observed in unfed larvae and larvae fed the combination of *T. chuii+C. muelleri*, while larvae fed all other diets did not differ in growth rates (Fig. 4.5). No difference was detected in larval mortalities (mean % mortalities ± SE, 2.6 ± 0.68%) among the diets ($F=2.295$, df 9,20, $P=0.058$).

Diet affected the percentage of eyed larvae produced (F=23.12, df 9,20, P<0.001). With the exception of the larvae fed T. Iso, larvae fed T. Iso+*T. chuii* produced an average 3.4 fold increase in the percentage of eyed larvae compared with larvae fed the remaining diets or unfed larvae (Fig. 4.6). A positive correlation between percentage eyed larvae and percentage of spat produced was detected (r=0.69, n=30, P<0.05).

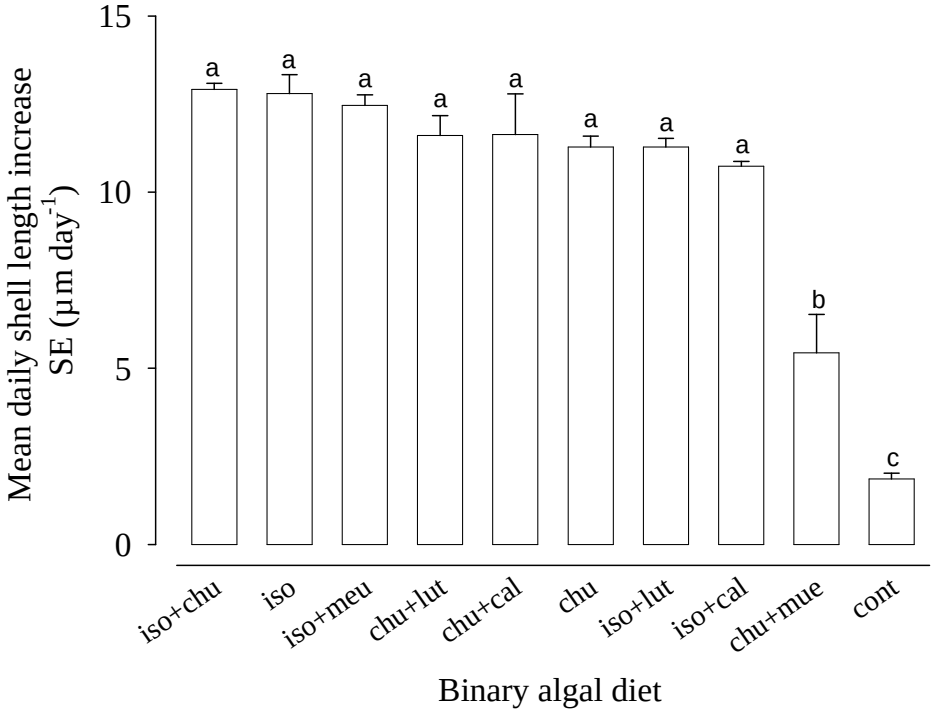

Figure 4.5 Mean daily growth rates in late stage *Ostrea angasi* larvae, previously fed binary algal diets (a pair wise cross of *Isochrysis* sp. (Tahitian) (iso) or *T. chuii* (chu) with either *P. lutheri* (lut), *C. muelleri* (mue) or *C. calcitrans* (cal) and unfed larvae (cont)). Means with the same letter are not significantly different from one another.

Diet affected the percentage of spat produced (F=8.72, df 9,20, P<0.001). Larvae fed T. Iso or T. Iso in combination with *T. chuii* or *C. muelleri* did not differ in the percentage of spat produced (Fig. 4.7). Larvae fed these diets produced an averaged 12.1 fold increase in the percentage of spat produced compared with larvae fed the remaining diets or unfed larvae. Larvae fed the remaining diets did not differ in the percentage of spat produced. A positive

correlation between growth rates and percentage of spat produced was detected (r=0.44, n=30, P=0.014).

Improvements in the larval growth rates, mean percentage eyed larvae and mean percentage of spat produced indicated the combination of *T. chuii*+T. Iso was the best binary algal diet for larvae during the late stage of larval development.

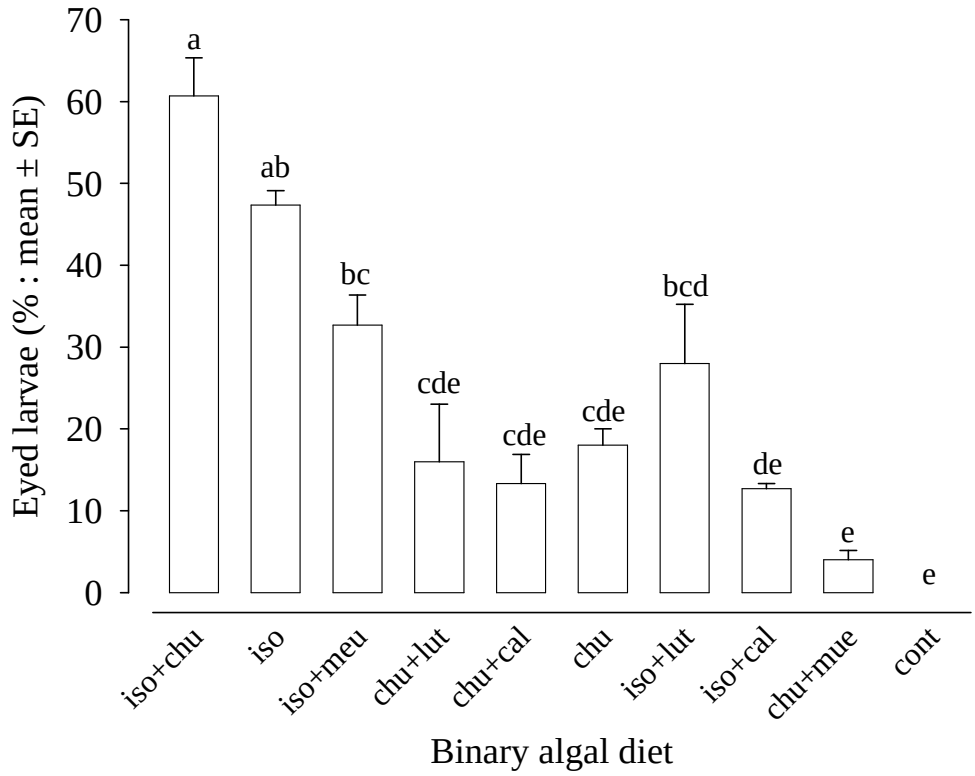

Figure 4.6 Mean *Ostrea angasi* eyed larvae (%) previously fed binary algal diets (a pair wise cross of *Isochrysis* sp. (Tahitian) (iso) or *T. chuii* (chu) with either *P. lutheri* (lut), *C. muelleri* (mue) or *C. calcitrans* (cal) and unfed larvae (cont)). Means with the same letter are not significantly different from one another.

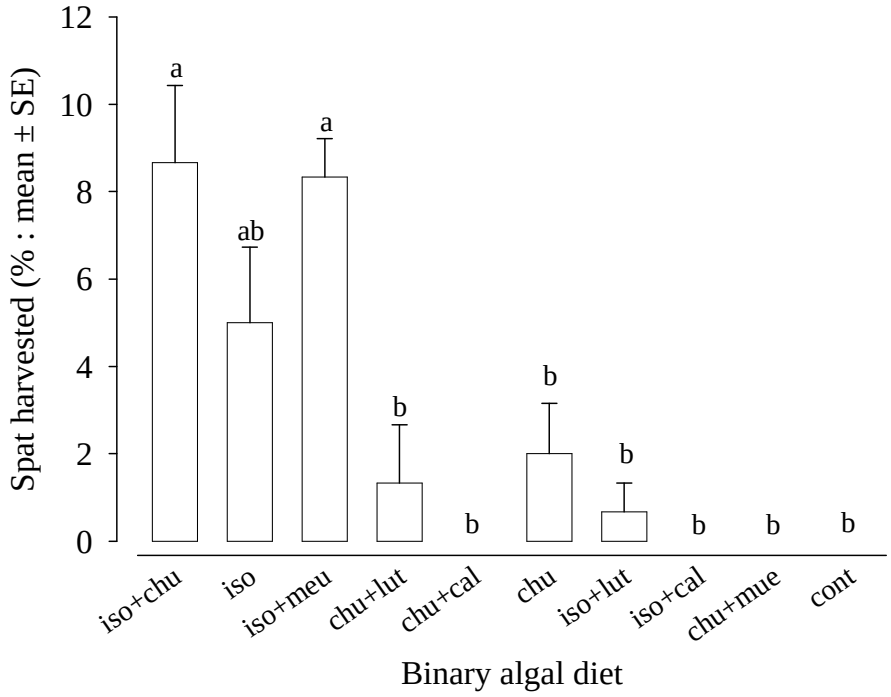

Figure 4.7 Mean *Ostrea angasi* spat (%) harvested from larvae previously fed binary algal diets (a pair wise cross of *Isochrysis* sp. (Tahitian) (iso) or *T. chuii* (chu) with either *P. lutheri* (lut), *C. muelleri* (mue) or *C. calcitrans* (cal) and unfed larvae (cont)). Means with the same letter are not significantly different from one another.

4.3.3 Ternary algal diet trial

Larval mortalities differed among diets (F=3.198, df 13,28, P=0.005). Larvae fed T. Iso+*P. lutheri*+*C. calcitrans* experienced a 7 fold increase in mortality rates compared to larvae fed T. Iso+*T. chuii*+*P. lutheri* diet. Mortalities among the remaining diets did not differ (mean% ± SE, 4.0±0.4%).

Larvae fed T. Iso+*T. chuii*+*P. lutheri* exhibited greater growth rates (F=33.04, df 13,28, P<0.001) than larvae fed the unialgal diets, unfed larvae and larvae fed either T. Iso+*T.*

chuii+C. muelleri or T. Iso+*P. lutherii+C. muelleri* but not differ significantly from larvae fed the remaining diets (Fig. 4.8).

The percentage of eyed larvae produced varied among diets (F=31.47, df 13,28, P<0.001). Larvae fed T. Iso+*T. chuii* with either *N. oculata* or *P. lutheri* resulted in an average 4 fold increase in the percentage of eyed larvae compared to larvae fed the remaining diets (Fig. 4.9).

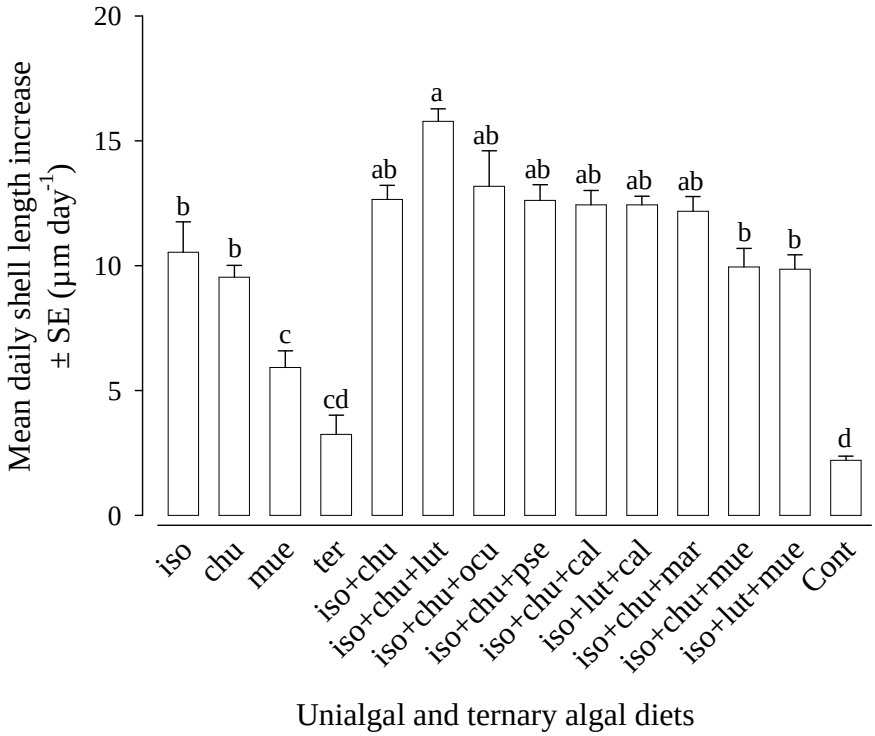

Figure 4.8 Mean daily growth in late stage *Ostrea angasi* larvae fed ternary algal diets (two algae: *Isochrysis* sp. (Tahitian) (iso) and *T. chuii* (chu), combined with either *N. oculata* (ocu), *P. lutheri* (lut), *C. muelleri* (mue), *C. calcitrans* (cal), *S. marinoi* (mar), *T. pseudonana* (pse)), binary diet single *Isochrysis* sp. (Tahitian) (iso) and *T. chuii*, uniaglal diets *Isochrysis* sp. (Tahitian) (iso), *T. chuii*, *C. muelleri* or *D. tertiolecta* (ter) and unfed larvae (cont)). Means with the same letter are not significantly different from one another.

Diets differed in the percentage of spat produced (F=9.54, df 13,28, P<0.001). Larvae fed T. Iso+*T.chuii* in combination with *N. oculata* or +*P. lutheri* were similar in the percentage of spat produced, but these diets produced a 15 and 13 fold increase respectively, over larvae fed the remaining diets or unfed larvae (Fig.4.10). A positive correlation was detected between *k* and the percentage of spat produced (*r*=0.44, n=42, P=0.003) and between percentage eyed larvae and percentage of spat produced (*r*=0.73, n=42, P<0.001). Larval growth rates and mean percentage of spat produced indicated the combination of *T. chuii* + T. Iso mixed with either *P. lutheri* or *N. oculata* was the best ternary algal diet for late stage larval development.

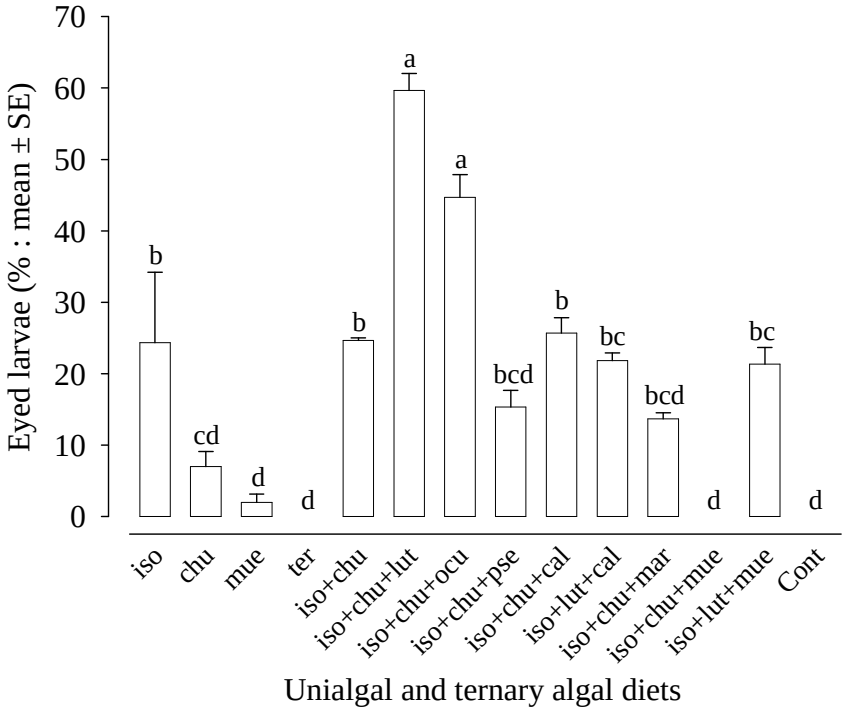

Figure 4.9 Mean *Ostrea angasi* eyed larvae (%) in late stage previously fed ternary algal diets (Two algae mixed, *Isochrysis* sp. (iso) and *T. chuii* (chu), combined with either *N. oculata* (ocu) , *P. lutheri* (lut), *C. muelleri* (mue), *C. calcitrans* (cal), *S. marinoi* (mar), *T. pseudonana* (pse), single species *Isochrysis* sp. (Tahitian) (iso), *T. chui*, *C. muelleri* or *D. tertiolecta* (ter) and unfed larvae (cont)). Means with the same letter are not significantly different from one another.

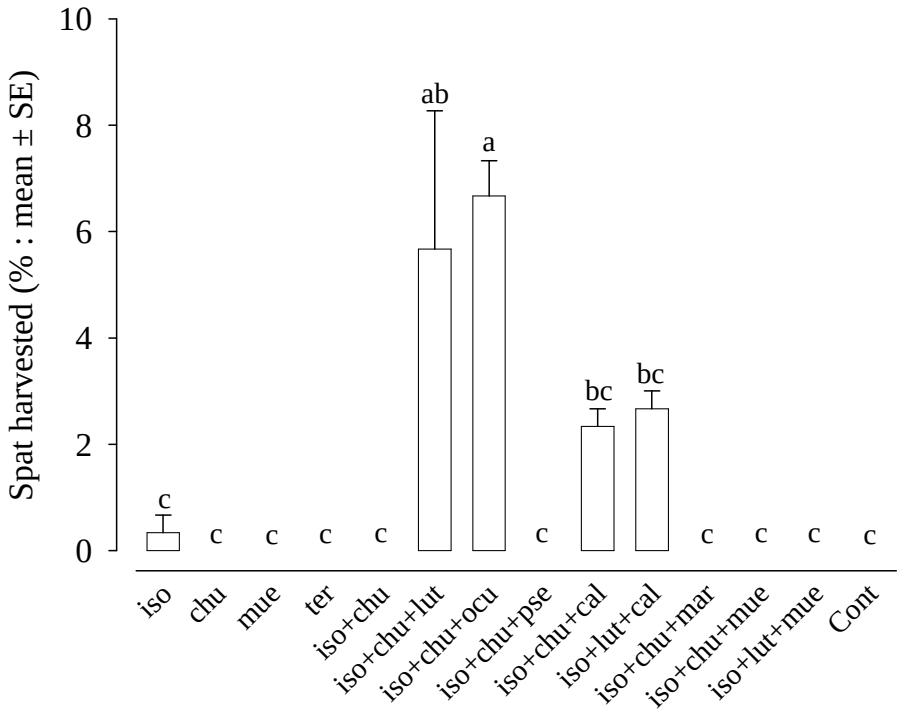

Figure 4.10 Mean *Ostrea angasi* spat (%) harvested from late stage larvae previously fed ternary algal diets (Two algae mixed, *Isochrysis* sp. (Tahitian) (iso) and *T. chuii* (chu), combined with either *N. oculata* (ocu), *P. lutheri* (lut), *C. muelleri* (mue), *C. calcitrans* (cal), *S. marinoi* (mar), *T. pseudonana* (pse), single species *Isochrysis* sp. (Tahitian) (iso), *T. chuii*, *C. muelleri* or *D. tertiolecta* (ter) and unfed larvae (cont)). Means with the same letter are not significantly different from one another.

4.4 Discussion

This study has shown that different microalgal diets affect *O. angasi* larval growth rates and development. The uni, binary and ternary algal diets fed to larvae resulting in the greatest percentage of spat produced were T. Iso, *T. chuii, N. oculata* or *P. lutheri*, T. Iso+*T. chuii*, T. Iso+*C. muelleri* and T. Iso+*T. chuii*+*P. lutheri* or T. Iso+*T. chuii*+*N. oculata*, respectively.

Additionally, it has been demonstrated that *O. angasi* larvae can ingest and digest algal cells ranging from 3µm to 15µm in size, the cell size of *N. oculata* and *T. chuii* respectively (table 4.1), with both algae providing good growth rates and development in the unialgal diet trials.

The effect of the unialgal diets on growth rates varied between early and late stages of larval development, with dietary effects more pronounced in the later stages of development. Bivalve larvae use parentally derived energy stored during gametogenesis to fuel the energetic demands of embryogenesis and early larval development (Collyer, 1957; Walne, 1965; Helm et al., 1973). In *O. angasi*, growth of early-stage unfed larvae indicates significant energy reserves were maternally provided, but the slower growth rates of late-stage unfed larvae suggest these reserves were depleted within the first eight days. Similarly, Labarta et al., (1999) found that the early stage of *O. edulis* larval development was both endo and mixotrophic, with larvae utilising maternally derived energy for up to 7 days and Walne (1965) indicated that *O. edulis* larvae are able to feed at a size slightly smaller than that at which they are normally released from the branchial chamber. Regardless, diets supporting the fastest growth during early stage development also produced the fastest, albeit slower growth, during the latter stage of larval development.

Greater uniformity of larval growth was observed among treatments as the algal diet gained complexity, but differences between diets occurred in the proportion of eyed larvae and spat produced. Helm (1977) found better growth and overall settlement of *O. edulis* larvae fed a mixed algal diet compared to a unialgal diet, yet in our study, little difference in growth rate was observed between the best uni, binary and the majority of the ternary diets trialed (compare Figs. 4.8, 4.9 & 4.10). This comparison of optimum uni and binary diets with the ternary diets indicates that larval growth rates are a relatively poor indicator of the ultimate success of a hatchery microalgal diet as the percentage spat produced was also affected by diet independently of growth rates, suggesting that the requirements for larval growth and competency to metamorphose may be affected by different dietary components. Larvae of *Pecten maximus* fed solely *C. calcitrans* or *S. costatum*, had good growth rates but these larvae failed to metamorphose (Delaunay et al., 1993). Likewise Tan Tui et al. (1989) indicated that altering diet regimes for *C. virginica* larvae affected development independent of growth rates. Although our dietary trials detected significant correlations between growth rates and percentage of spat produced in each trial, the strength of the correlation declined

with dietary complexity. The significant correlation between the percentage of eyed larvae and the percentage of spat produced observed in this study could allow assessment of eyed larvae as an indicator for dietary value without needing to undertake larval settlement.

The greatest larval growth rates obtained in the uni, binary and ternary diet trials were 11.2, 12.9 and 15.7µm day^{-1} respectively. These growth rates exceed that obtained by Dix (1976) for *O. angasi* larvae fed an algal diet *Isochrysis galbana*, *P. lutheri* and *Tetraselmis suecica* of 8µm day^{-1} at a larval rearing temperature of 17^{o}C or that obtained by O'Sullivan (1980) for *O. angasi* larvae fed an algal diet of *P. lutheri* of a maximum of 5.8µm day^{-1} at a larval rearing temperature of 15^{o}C and are comparable with that obtained by Hickman and O'Meley (1988 a & b) for *O. angasi* larvae fed an algal diet of T. Iso and *P. lutheri* of a maximum 11.7µm day^{-1} at a larval rearing temperature of 21^{o}C. However, the different larval growth rates obtained are likely to be a combination of both algal diet and larval rearing temperature affecting growth rates. The average percentage mortality obtained in the unialgal diet trials were higher than that recorded by Dix (1976) (of less than 10% mortalities). The differing levels of mortalities observed between these two trials, may be due differences in egg quality, lack of dietary requirements for good growth and survival in some single algal species used or poor ingestion and digestion. Differences observed in larval growth, survival and development between algal diets may also reflect the nutritional status of the female oyster during vitellogenesis (Millican and Helm 1994; Bertsson et al., 1997).

Of the unialgal diets fed to *O. angasi* larvae it can be concluded that larvae both ingest and digest T. Iso, *N. oculata*, *P. lutheri*, *T. chuii*, *C. muelleri* and *S. marinoi* as indicated by appreciable growth at least seven fold greater than the unfed larvae (Fig. 4.2) with no difference in larval mortalities between diets (Fig. 4.1). This does not indicate nutritional suitability of all these algal species as a uinialgal diet for *O. angasi* larvae, only that larval growth and development to eyed larvae occurred when fed these algal species. *C. calcitrans* can be discounted as having any toxic exudates as comparable survival, growth and development were observed when this algae and supernatant were combined in the larval diet with either T. Iso or *T. chuii* as when larvae were fed either of the latter two flagellates alone. Additionally, algal cultures were assessed for the presence of potential pathogenic bacteria no assessment for the presence of beneficial strains was conducted. The presence of beneficial bacterial strains giving better performance of larvae on a specific algal diet should not be discounted (Leroy et al., 2012)

Table 4.1. Cell size, CSIRO* code and arachidonic acid (20:4(n-6)), eicosapentaenoic acid (20:5(n-3)), docosahexaenoic acid (22:6(n-3)), docosapentaenoic acid (22:5(N-6)) composition of algal species used for the dietary trials.

Species	CSIRO* code	Size μm	Fatty Acids			
			20:4(n-6)[a]	20:5(n-3)[a]	22:5(n-6)[b]	22:6(n-3)[a]
Isochrysis sp. (T.*Iso*)	CS-177	3 x 5	≤ 0.2%	≤ 0.2%	1-5%	>20%
Pavlova lutheri	CS-182	4 x 6	0.2-1%	>20%	1-5%	5-20%
Tetraselmis chuii	CS-26	15 x 9	0.2-1%	5-20%	NA	≤ 0.2%
Chaetoceros muelleri	CS-176	5-8	≤ 0.2%	5-20%	≤ 0.2%	0.2-1%
Chaetoceros calcitrans	CS-178	3-6	0.2-1%	5-20%	≤ 0.2%	0.2-1%
Skeletonema marinoi	CS-181	10x5	≤ 0.2%	5-20%	≤ 0.2%	1-5%
Thalassiosira pseudonana	CS-173	4-5	0.2-1%	5-20%	≤ 0.2%	1-5%
Nannochloropsis oculata	CS- 179/4	3	5-20%	>20%	NA	≤ 0.2%
Dunaliella tertiolecta	CS-175	10-12	≤ 0.2%	≤ 0.2%	≤ 0.2%	≤ 0.2%

a. extracted from Brown et al., 1997; b. extracted from Brown et al., 1989.

* CSIRO: Commonwealth Scientific and Industrial Research Organisation (Australia)

The unialgal diets in this study shed some light on the nutritional needs of *O. angasi* larvae. As growth rates and mortalities did not differ significantly among larvae fed T. Iso. (low arachidonic acid (AA) & eicosapentaenoic acid (EPA) & high docosahexaenoic acid (DHA) levels), *T. chuii* (high EPA, low AA & DHA levels), *N. oculata* (high AA & EPA, low DHA

levels) or *P. lutheri* (Low AA, high EPA & DHA), it is possible that *O. angasi* larvae may have a requirement for either EPA or DHA, but may not require both in the diet (Table 1). High levels of EPA in the diet have been negatively correlated with growth in both *C. gigas* larvae (Thompson et al., 1996) and *O. edulis* larvae (Jonsson et al., 1999) and low levels of DHA have a negative effect on metamorphosis of *P. magellanicus* larvae (Pernet et al., 2005). High or low dietary levels of either EPA, DHA or AA did not appear to affect survival, growth or development of *O. angasi* larvae. Though separate functions have been allocated for these PUFA's it has been suggested that EPA and DHA can substitute to meet the dietary requirements for the n-3 PUFA for *C. gigas* (Langdon and Waldock, 1981), and this would also appear to be the case for *O. angasi* larvae. The poor dietary performance of the diatoms used in this trial was unexpected, though ingestion and digestion of *C. muelleri* and *S. marinoi* can be inferred this is not the case for *C. calcitrans* or *T. pseudonana*. In our study, the effect of dietary PUFA's requires determination of the origin (parentally or dietary derived), accumulation, and fate of these essential fatty acids.

For hatchery production of *O. angasi* larvae, ternary diets of *T. chuii*+*T.* Iso combined with either *P. lutheri* or *N. oculata* were the best diets based on rates of larval growth rates, survival and development. The use of Epi-B treatment of larvae allowed easy quantification and assessment of dietary impacts on the rate of development of competency and maturation of *O. angasi* larvae. The timing for Epi-B treatments in our experiments were intended to examine the effects of diet on the onset of competency, with the total number of larvae successfully metamorphosing on the different diets to be examined at a later date. Hence the relatively low percentage of induced larval metamorphosis encountered in these experiments is due to experimental rearing protocols (ie. no culling of slow growing individuals during larval rearing, a common hatchery practice to achieve greater larval developmental uniformity at metamorphosis) and the stage of larval development at which larvae were exposed to epinephrine treatment. To discern dietary effects on onset of competency, larvae were exposed to the morphogenic agent at an earlier stage of larval development than recommended for hatchery production of this species (O'Connor et al., 2009). This study of *O. angasi* larval dietary requirements suggest that further work examining the source and fate of essential PUFAs is worthy of further investigation, and that other dietary factors play a role in *O. angasi* larval development and metamorphosis.

Chapter 5: Assessment of temperature or salinity effects on larval development by catecholamine induced metamorphosis of hatchery reared flat oyster, *Ostrea angasi* (Sowerby 1871) larvae.

5.1 Introduction

Successful hatchery production of bivalves depends on an understanding of larval and spat physiology and the impact of environmental factors on rate of larval growth, survival, and development. Metamorphosis in particular, is a critical transitional phase during bivalve larval development when substantial variability in metamorphosis and mortality occurs (Rico-Villa et al., 2006). Aside from larval diet, rearing temperature (Rico-Villa et al., 2009; Kheder et al., 2010) and salinity (His et al., 1989; Zimmerman and Pechenik, 1991; Dove and O'Connor, 2007) are important factors affecting larval metamorphosis and survival. In hatchery production of bivalve larvae the rearing environment can be altered to maximise production. Accordingly, assessment of the effects of these environmental parameters on hatchery production of any bivalve species warrants investigation (Holland and Spencer, 1973; Gallager et al., 1986; Devakie and Ali, 2000).

The effect of changing larval rearing temperature on survival and metamorphosis of competent mollusc larva varies among mollusc species. When rearing temperature of competent *Crassostrea virginica* larvae was elevated by 5^{o}C for 1h a significant increase in the rate of larval metamorphosis occurred (Lutz et al., 1970). Alternatively, Davis and Calabrese (1969) suggested that rearing temperature for *Ostrea edulis* larvae be reduced from 27.5^{o}C, to 25^{o}C or 20^{o}C at metamorphosis to increase the number of spat produced. However, if settlement inducers, such as catecholamines are to be applied, the influence of change in larval rearing temperature on successful metamorphosis may alter. Lowering larval rearing temperature for *Crassostrea gigas* decreased the metamorphic induction of L-Dopa on competent larvae (Cooper and Shaw, 1983). For this reason, hatcheries intending to use chemical induction of larval metamorphosis need to assess the effect of temperature fluctuation during this sensitive stage of larval development.

If settlement protocols are to be developed, it is important that their outcomes are repeatable between different larval cohorts. Studies with *C. gigas* have shown the settlement success of different cohorts of larvae have significant genotype by environment interactions (Taris

et al., 2007) and high genotype dependent mortalities can occur during metamorphosis (Plough and Hedgecock, 2012). Further, larvae with different environmental histories that are reared in the same conditions for the remainder of their larval life cycle may react differently to the same settlement protocols. This is particularly important for larvae from brooding genera such as the *Ostreinae*, where larvae are frequently collected from comparatively small numbers of brooding adults and brought to the hatchery to complete their larval development. Some genetic variation has been found among cohorts of *Ostreinae* larvae (Hedgecock et al., 2007; Taris et al., 2009) which may influence larval survival and metamorphosis. If comparatively small numbers of brood-stock contribute to the genetic variability of a larval cohort, larval metamorphosis could vary depending on the protocols applied. It is therefore important to assess the repeatability of settlement protocols across larval cohorts.

Internationally, interest in the hatchery production of Ostried "flat" oysters is increasing and there is a renewed focus on improving hatchery techniques (Kesarcodi-Watson et al., 2012; Martinez Castro, 2013). In Australia, this is true for the native or flat oyster, *O. angasi*, where the vast majority of seed production is hatchery reliant. To date, different diets and rearing temperature have been used to produce *O. angasi* larvae (Dix, 1976; O'Sullivan, 1980; Hickman and O'Meley, 1988b; O'Connor et al., 2012), but there is little information regarding the effects of temperatures and salinity on larval growth, survival and metamorphosis. In particular, little attention has been paid to the effects of changes in larval rearing temperature and its effects during chemical induced metamorphosis. The aims of this study were to firstly improve *O. angasi* rearing protocols by determining the effects of different rearing temperatures or salinities on larval growth, survival, development using catecholamine induced metamorphosis. Secondly, examine the effects of short term alteration of larval rearing temperature in combination with catecholamine induced metamorphosis.

5.2 Materials and Methods

5.2.1 Larval collection

Brood-stock *O. angasi* were collected from wild populations at Wagonga Inlet, Narooma (36^{o} 12' 22.99" S 150^{o} 25' 53.90" E), Bermagui River (36^{o} 25' 42.61" S 150^{o} 03' 18.80" E), Merimbula Lake (36^{o} 54' 07.72" S 149^{o} 52' 42.77" E) on the south coast of NSW or Camden Haven River (31^{o} 38' 36.39" S 152^{o} 49' 54" E) on the mid north coast of NSW. Oysters were

narcotised (MgCl [50gm L^{-1}] in 25% sea water-75% freshwater (Butt et al., 2008) and the brood of larvae rinsed from the pallial cavity into separate 1L plastic beakers. Each brood of larvae was rinsed thoroughly in 1µm filtered seawater, counted, sized and the stage of larval development determined. Each experiment used larvae of a similar size and stage of development pooled from at least three larval broods (the combination of separate larval broods is referred to as a larval cohort). All larvae were reared at a density of 2 larvae mL^{-1} in 120mL polypropylene containers (Techno Plas S10844-04) using 100mL of 1µm filtered seawater, at a salinity of 35 (FSW), unless stated otherwise.

5.2.2 Larval rearing

Stable temperatures were maintained by placing the 120mL containers in recesses in an aluminium block with thermal control (± 0.5^{o}C); similar to the apparatus employed by Kennedy et al., (1974) having a controlled heating and cooling applied to opposite ends of the aluminium block giving a stable temperature gradient in along the longitudinal axis of the aluminium block. The water in each larval rearing container was changed daily and larvae were fed twice daily with a diet of Tahitian *Isochrysis* aff. *galbana* and *Tetraselmis chuii* on an equal dry weight basis (Nell and O'Connor, 1991) using a standard feed curve developed for Sydney rock oyster larvae (O'Connor et al., 2008). The algal species used to feed the larvae were selected because they tolerate the range of temperatures and salinities used in this experiment (Renaud and Parry, 1994; Nelo Medeiros Perfeto etal., 1997) and because they support good growth and development of *O. angasi* larvae (O'Connor et al., 2012).

During each experiment the time at which larvae attained competency and the ability to undergo metamorphosis, was identified using a reference batch of larvae that were concurrently cultured in standard rearing conditions employed at the NSW Port Stephens Research Institute Mollusc Hatchery, in FSW at 26^{o}C, in 120mL containers. Larvae were defined as being competent to undergo metamorphosis once they reached a shell length of approximately 340-350µm, had an eye spot and a ciliated foot present, had developed 4-5 rudimentary gill filaments, and had an extendable foot and/or they were crawling. When the reference batch of larvae had reached competency a sample of larvae was collected from each replicate container in the experiment. Survival was determined by counting the number of live larvae among the first 100 larvae viewed (100x magnification), while the average

dorso-ventral shell length was determined by measuring the first 30 larvae observed. Average daily specific daily growth rates (*k*) were calculated using the average shell length data as follows:

$$k = (\ln SL_1 - \ln SL_0) / t_t$$

where SL_1 is shell length at the end of cultivation time t_t (days) and SL_0 is the initial shell length (antero-posterior measurement) at start of the trial. The remaining larvae in each replicate container were then treated with 10^{-3} molar epinephrine bitartrate (Epi-B, Sigma-Aldrich, # E4375) for 1h (O'Connor et al., 2009), the number of Epi-B treatments varying depending upon the experiment. Following final treatment, larvae were returned to their rearing containers in FSW for a further 24h before being fixed with 10% formalin in seawater for later examination. From the first 100 larvae viewed, the number of eyed larvae, dead larvae and spat were determined. Metamorphosed larvae (spat) were determined by examination at 400x and 200x magnification using a Leica DME light microscope for the loss of the velum and the initiation of the production of the dissoconch shell. Larval mortality was determined from the number of empty larval shells or larvae with tissue necrosis.

5.2.3 Effect of salinity on larval growth, development and mortality

Larvae with a mean starting shell length of 280 ± 10μm (mean ± SD), were reared at five different salinities (15, 20, 25, 30, and 35) to determine the effect of salinity on larval growth, survival and percentage of spat produced. Salinity was adjusted by diluting FSW with deionised freshwater to obtain the required salinities and measured with a calibrated Yeo-Kal 605 Dip Meter (Yeo-Kal Electronics P/L, Sydney, Australia). Water exchanges and feeding was carried out daily. To ensure salinity remained constant within treatments the algal ration (adjusted for dilution factor to maintain a constant ration across treatments) was added prior to dilution with FSW. To minimise osmotic shock, larvae were acclimated to the required salinity by decreasing the salinity at a rate of 5ppt day^{-1}. Larvae (n=5 replicate rearing containers) were reared for a total of nine days; four days for the acclimation process (those larvae already at the required salinity had water exchanges the same as those larvae still being acclimated), then five days at their respective test salinities at 26^{o}C. At the end of the nine days, at least 30 larvae from each replicate container were fixed in 10% formalin to determine growth rates. The remaining larvae were treated with Epi-B once every 24h, for three

consecutive days. Twenty-four hours after the final Epi-B treatment larvae were fixed for examination of growth, survival and percentage metamorphosis.

5.2.4 Effect of larval rearing temperature on larval growth, mortality and development

To examine the effect of temperature on larval growth and development, larvae (mean shell length ± SD: 155 ± 6μm, n=3 replicate rearing containers) were reared at seven different temperatures, 14.5, 18, 20.5, 23, 26, 29 or 31^{o}C ± 0.5^{o}C. Larvae were acclimated from 25.5^{o}C to the desired rearing temperature over a 24h period then reared for a further 12 days. At the end of this period all larvae were fixed in 10% formalin for examination of growth, survival and percentage eyed larvae.

5.2.5 Effect of larval rearing temperature on onset of larval competency and mortality

To determine the interactive effect of larval rearing temperature and Epi-B on the onset of larval competency, larvae (n=6 replicate rearing containers) were reared at five different temperatures, 20.5, 23, 26, 29 or 31^{o}C ± 0.5^{o}C, from a mean shell length ± SD: 190 ± 13μm, until competency was observed in larvae reared at 26^{o}C (larval rearing period of 11 days). Subsequently, larvae were maintained at their respective rearing temperatures and exposed to two treatments of either Epi-B or FSW for 1h every 24h for three consecutive days. Larvae were fixed for examination of survival and metamorphosed larvae 24h after the third day of treatment.

5.2.6 Effect of temperature manipulation and epinephrine bitartrate treatment on larval and metamorphosis

To determine the effect of manipulating larval rearing temperature, alone and in conjunction with Epi-B treatment, on survival and metamorphosis of competent larvae, two separate cohorts (cohort) of larvae from different estuaries (Bermagui and Laurieton) were reared to competency using the standard larval rearing protocols. Previously, short term decrease (1h) of larval rearing temperature from 26^{o}C to 20.5^{o}C increased *O. angasi* larval mortalities (unpublished data). Therefore, it was decided that the minimum temperature for this experiment would be 23.5^{o}C. Two separate cohorts of larvae (mean shell length ± SD: 358 ± 5μm, n=3 replicate rearing containers) had the temperature in their rearing container altered from 26^{o}C to either of six different temperatures, 23.5, 25.5, 26, 27.5 29.5 or 31.5^{o}C, for 1h and simultaneous exposure to a catecholamine, either Epi-B or FSW for 1h. At the end of the

1h treatment period larvae were returned to FSW at 26^{o}C for a further 24h before being fixed for examination of survival and metamorphosis.

5.2.7 Statistical Analysis

All analyses were conducted using SPSS v.16.0 software (SPSS Inc. 2007, Chicago Illinios, USA). Data was analysed with one-way or two-way ANOVA to determine if any significant difference existed between treatments. Where significant differences were found, pairwise comparisons among the means were made using Tukeys HSD (α = 0.05). In two-way ANOVA where significant interaction were detected between independent variables, the independent variables were pooled for *Post Hoc* comparison (Tukeys HSD, α = 0.05). All mean values, unless stated otherwise, are ± standard error (SE).

5.3. Results

5.3.1 Effect of salinity on larval growth, mortality and development

Salinity significantly affected larval growth ($F=61.4789$, df 4,20, $P<0.001$), with larvae reared at 25, 30 or 35 growing (mean shell length increase: 8 ± 3µm day^{-1}) , 1.5 and 3.5 times faster than larvae reared at a salinity of 20 (5 ± 0.3µm day^{-1}) and 15 (2.3 ± 0.4µm day^{-1}) respectively (Fig. 5.1). The salinity in which larvae were reared affected larval metamorphosis into spat ($F=29.468$, df 4,20, $P<0.001$). The greatest number of spat were produced at the higher salinities (35 and 30; mean % spat produced: 56.4 ±7.6 and 63 ± 8 respectively) produced approximately 2 and 15 times more spat compared to larvae reared at a salinity of 25 (25.5 ± 4.2%) or 20 (4.2 ± 1.8%) respectively (Fig. 5.1). Larvae reared at a salinity of 15 did not successfully metamorphose to spat.

The percentage of larval mortality was also affected by salinity ($F=98.863$, df 4,20, $P<0.001$), but only in the least saline conditions. Larvae reared at a salinity of 15 experienced 15 times more mortality (mean % mortality: 83.4 ± 4.7%) than larvae reared at salinities of 20, 25, 30 and 35 (0.2 ± 0.2, 0.8 ± 0.5, 1.4 ± 0.6 and 0.6 ± 0.4 respectively).

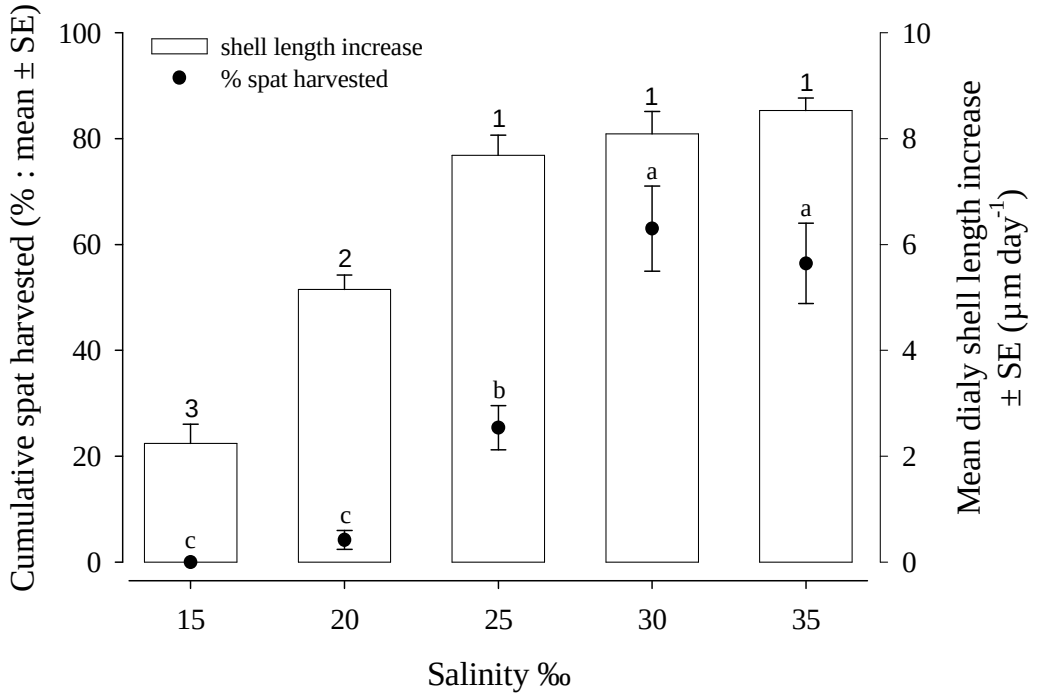

Figure 5.1 Mean daily shell length increase and cumulative percentage spat produced from *Ostrea angasi* larvae reared at salinities of 26°C at 15, 20, 25, 30 or 35ppt. When competency was observed at a salinity of 35 larvae where treated with either 10^{-3} molar epinephrine bitartrate(Epi-B) or fresh filtered sea water for 1h, daily for three consecutive days. Means with the same letter or number are not significantly different from one another.

5.3.2 Effect of larval rearing temperature on larval growth, mortality and development

Temperature significantly affected mean larval growth rate ($F=116.49$, df 6,14, $P<0.001$) and mean percentage yield of eyed larvae ($F=13.99$, df 6,14, $P<0.001$). Larval growth rate at 26 and 29°C (mean shell length increase µm day^{-1}: 12.4 ± 0.3µm day^{-1} and 13.2 ± 0.4µm day^{-1} respectively) was five-fold faster than the larval growth rate at 14°C (2.4 ± 0.1µm day^{-1}) (Fig. 5.2). Larval growth rate declined at 31°C (10.9 ± 0.4µm day^{-1}). The yield of eyed larvae generally reflected larval growth rate, with maximum percentage eyed larvae obtained at 26 and 29°C (% eyed larvae: 7 ± 0.6% and 6.7 ± 1.2% respectively) (Fig. 5.2).

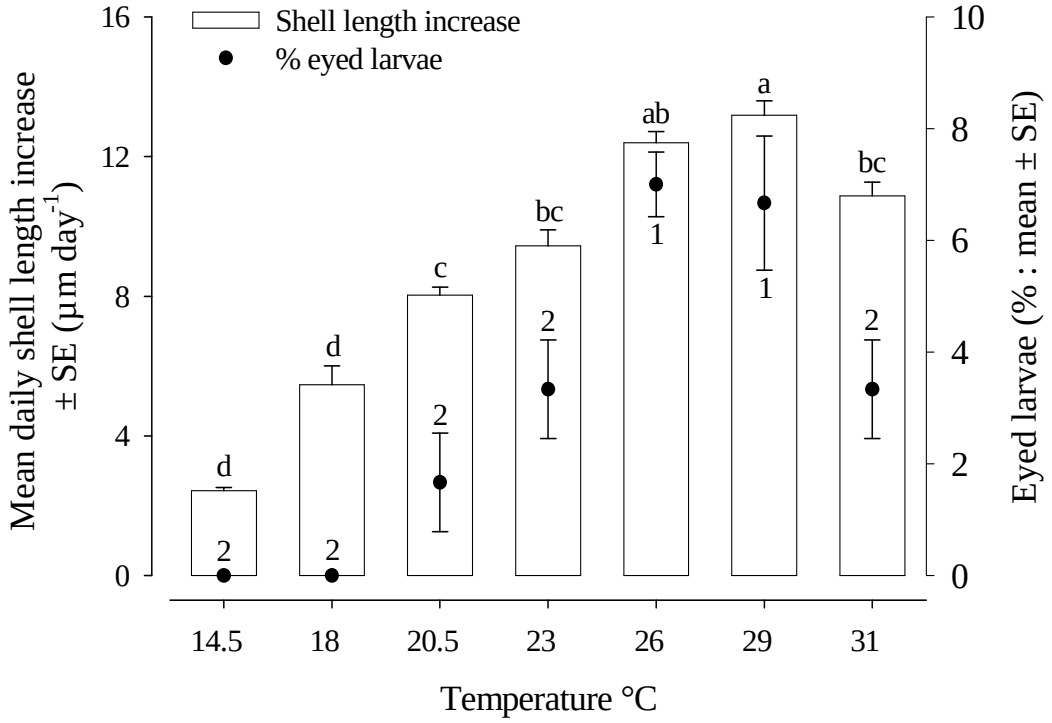

Figure 5.2 Mean daily shell length increase and percentage eyed larvae from *Ostrea angasi* reared at either 14.5, 18, 20.5, 23, 26, 29 or 31°C. Means with the same letter or number are not significantly different from one another.

Larval mortality was significantly affected by rearing temperature (F=7.28, df 6,14, P=0.001). Larvae reared at 14.5, 18, 20.5, 23, 26 and 29°C did not differ in mortalities (mean % larval mortality: 3 ± 0.58, 1.3 ± 0.33, 1 ± 0.57, 1 ± 0, 1 ± 0.33 and 2.7 ± 0.33 respectively). Larvae reared at 31°C (2.7 ± 0.3%) did not differ in mortality from larvae reared at 14.5 or 29°C but larvae reared at 31°C had significantly greater mortality than larvae reared between 18 to 26°C.

5.3.3 Effect of larval rearing temperature on onset of larval competency and mortality

The percentage of spat produced varied significantly with larval rearing temperature, but the differences among the temperatures depended on whether larvae were treated with or without Epi ($F_{temperature*treatment}$=13.16, df 4,40, P<0.001). Larvae reared at 26, 29 or 31°C produced significantly more spat when treated with Epi-B (mean % spat produced: 42.6 ± 2.25%, 49 ± 2.1% and 35.7 ± 3.1% respectively) compared with no treated larvae reared at the same temperature (17.3 ± 2.5%, 18 ± 2.5% and 15.9 ± 1.9% respectively). However, larvae reared

at 20.5 and 23°C did not differ in the amount of spat produced whether treated with Epi-B or FSW (Epi-B: 11.6 ± 1.2%, 17.4 ± 1.7%; FSW: 5.8 ± 1.2% and 7.2 ± 0.8% respectively) (Fig. 5.3).

Mean percentage larval mortalities were consistent over the rearing temperature range tested and treatment (with or without Epi-B treatment) combined ($F_{temperature*treatment}$=4.402, df 4,40, P=0.066) and there was no evidence that epinephrine bitartrate treatment alone effected larval mortalities (F=0.546, df 1,40, P=0.464). However, larval rearing temperature had a significant effect on larval mortality (F=13.06, df 4,40, P<0.001). Mortality among larvae reared at 31°C (mean % larval mortality pooled for Epi-B treatment: 13.7 ± 2.5%) was on average 4.5 fold greater larvae reared at temperatures of 20.5 23, 26 or 29°C (2.7 ± 1.5, 2.2 ± 0.8, 4.15 ± 1.3 and 3.25 ± 0.9 respectively).

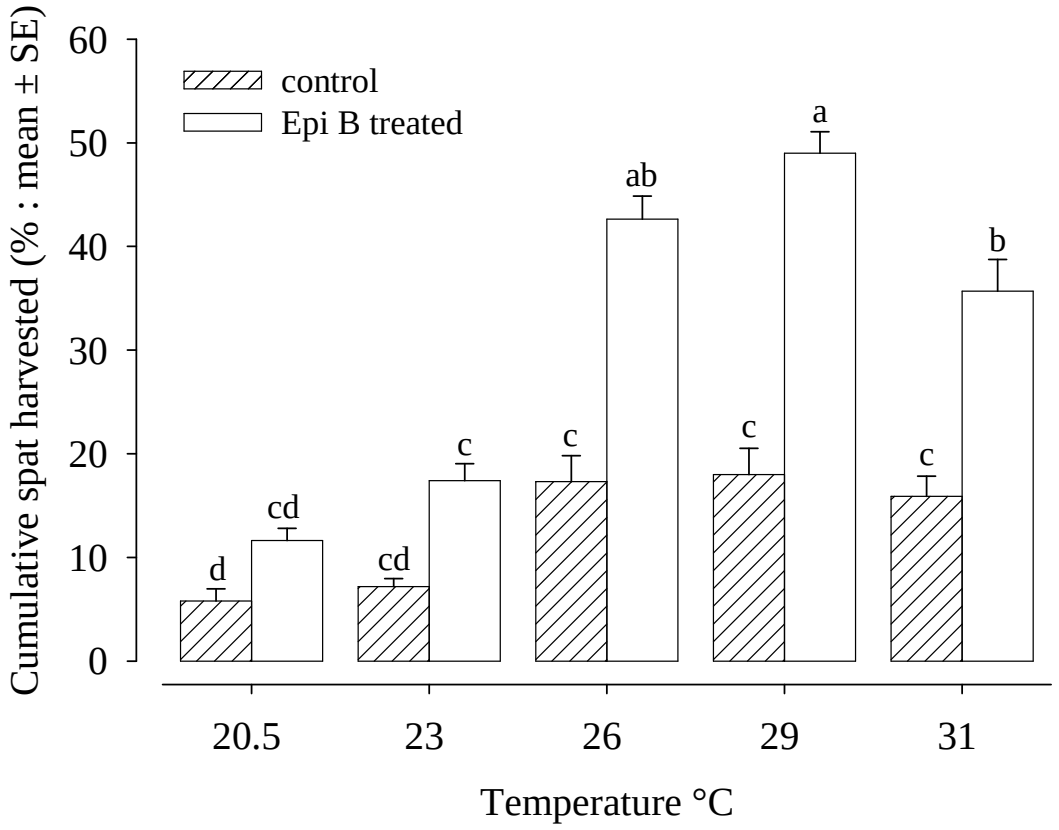

Figure 5.3 Mean cumulative percentage of spat harvested from *Ostrea angasi* larvae reared at 20.5, 23, 26, 29 or 31°C to competency and treated with either 10^{-3} molar epinephrine bitartrate (Epi-B) or fresh filtered sea water for 1h, daily for three consecutive days. Means with the same letter are not significantly different from one another.

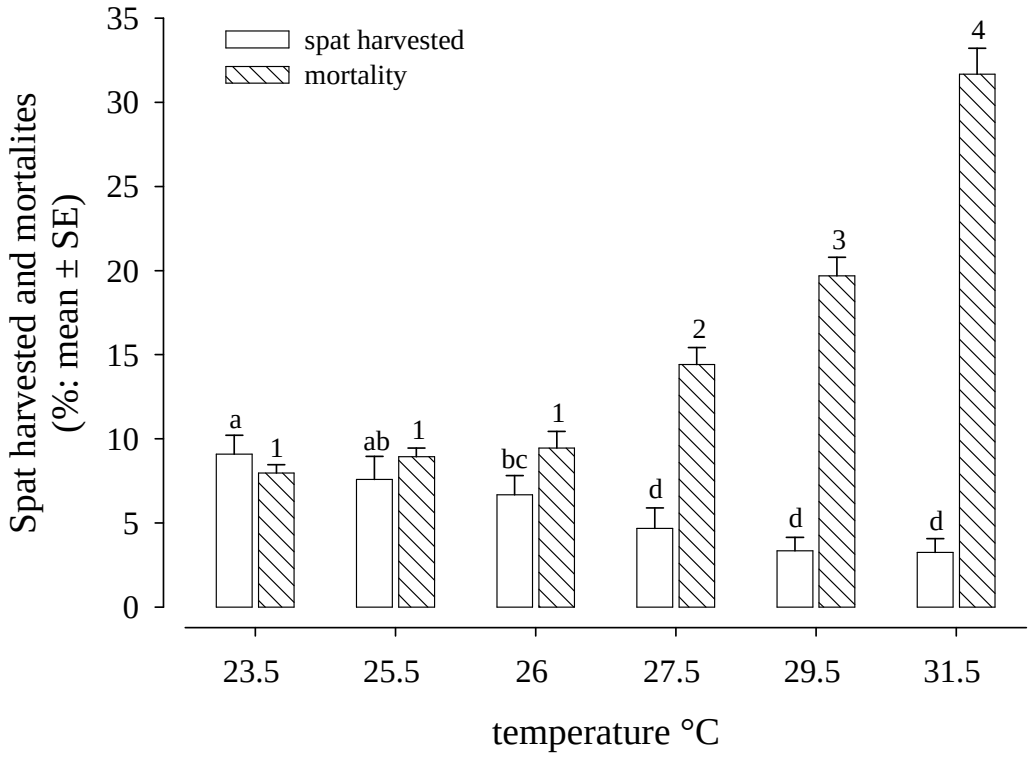

Figure 5.4 Mean percentage of *Ostrea angasi* spat harvested and mortality from larvae reared at 26°C, until larvae exhibit signs of competency and then the rearing temperature altered to either 23.5, 25.5, 27.5, 29.5 or 31.5°C once, for 1h (pooled for larval cohort and Epi-B treatment). Means with the same letter (spat harvested) or number (mortality) are not significantly different from one another.

5.3.4 Effect of temperature manipulation and epinephrine bitartrate treatment on larval mortality and metamorphosis

The combination of all three factors, temperature manipulation (TM) , Epi-B treatment and larval cohort ($F_{TM*treatment*cohort}$ =1.25, df 5,48, P=0.300), a combination of TM and larval cohort ($F_{TM*cohort}$ =1.78, df 5,48, P=0.272), a combination of Epi-B treatment and larval cohort ($F_{treatment*cohort}$ =0.48, df 1,48 P=0.520) or a combination of Epi-B treatment and TM ($F_{treatment*TM}$ =1.32, df 5,48, P= 0.383) did not affect the percentage spat produced. Short term

manipulation larval rearing temperature affected the percentage of spat produced (F =10.98, df 5,48, P=0.01) (Fig. 5.4). With a greater percentage of spat produced with decreasing temperature from a base rearing temperature of 26^{o}C to 23.5^{o}C (mean % spat produced: 9.1 ± 0.8%) and lower percentage of spat produced with increasing temperature from 26^{o}C to 31.5^{o}C (3.3 ± 0.5%). Larvae treated with Epi-B produced (8.8 ± 0.7%) significantly greater percentage of spat compared to non-treated larvae (2.3 ± 0.5%) (F=433.4, df 1,48, P=0.31). The percentage spat produced did not differ between the larval cohorts (F=5.29, df 1,48, P=0.190).

Larval mortalities were not affected by a combination of either Epi-B treatment, TM and larval cohort ($F_{treatment*TM*cohort}$=1.152, df 5,48, P=0.347), TM and larval cohort ($F_{TM*cohort}$=1.86, df 5,48, P=0.256), Epi-B treatment and larval cohort ($F_{treatment*cohort}$=0.234, df 1,48, P=0.65) or Epi-B treatment and TM ($F_{treatment*TM}$=1.288, df 5,48, P=0.394). Significant variation in mean percentage larval mortalities occurred with TM (F=51.18, df 5,48, p<0.001), with lower mortality occurring with decreasing temperature 23.5^{o}C (2 ± 0.32%) from a base rearing temperature of 26^{o}C (3 ± 0.87%) and higher mortality occurring with increasing temperature to 31.5^{o}C (27.92 ± 2.96%) from the same base temperature (Fig. 5.4). Neither Epi-B treatment or different larval cohorts affected mortality (F=39.27, df 1,48, P=0.103 and F=5.14, df 1,48, P=0.221 respectively).

Temperature and salinity are significant factors affecting survival, growth and metamorphosis of *O. angasi* larvae. The fastest larval growth rates were obtained at temperatures and salinities of 26-29^{o}C (mean shell length increase μm day^{-1}: 17.7 ± 3.5μm day^{-1}) and 25-35 (8.1 ± 3.3μm day^{-1}) respectively. The fastest rate of larval development and greatest percentage of spat produced occurred between 26-29^{o}C (mean % spat produced: Epi-B treated: 45.8 ± 2.1%; FSW treated: 17.7 ± 2.5%) corresponding with best growth rates. Short term (1h) changes in larval rearing temperature during metamorphosis affected the number of spat produced and larval mortalities. Decreasing temperature during catecholamine induced metamorphosis, from 26^{o}C to 25.5^{o}C or 23.5^{o}C (mean % spat produced: 11.5 ± 1.2% and 12.2 ± 1% respectively), increased spat production without affecting larval mortality.

5.4 Discussion

Given the sub-tidal, stenohaline habitat of *O. angasi* (Tompson, 1957) it is not surprising that the greatest larval growth was recorded between salinities from 25 and 35, with a decline in growth rates with salinities at or below 20. Nell and Gibbs (1986) reported comparable tolerances for *O. angasi* spat with no survival below a salinity of 20 and best growth between salinities of 25 to 35. A similar response to less saline conditions occurred in hatchery reared *O. edulis* larvae, with greater mortality and slower larval growth at a salinity of 15 and reduced growth rates and lower numbers of spat produced at a salinity of 20 when compared to larvae reared at higher salinities (Davis and Ansell, 1962). Though growth rates of *O. angasi* larvae did not differ across salinities of 25 to 35, a significant reduction in the percentage of spat produced occurred among larvae reared at salinity of 25 or less, over the same larval rearing period. A comparable response was found with *Crepidula plana* where larvae had similar growth rates at different salinities but were slower to attain competency at lower salinities tested (Zimmerman and Pechenik, 1991).

The larval growth rates observed in this study were slower than recorded in a number of studies; O'Sullivan (1980) reported 5.8µm day^{-1} at 15^{o}C (this study ≈3µm day^{-1}), Dix (1976) reported 8µm day^{-1} at 17^{o}C (this study ≈5µm day^{-1}) and Hickman and O'Meley (1988b) reported 11.7µm day^{-1} at 21^{o}C (this study ≈8.5µm day^{-1}). However, no information is available for a comparison of growth rates at warmer temperatures for this species and given the possible genetic variation between geographically distant populations of the same species (Koehn et al., 1976). Larval growth rates increased with rising temperature from 14.5^{o}C to 29^{o}C, but decreased at 31^{o}C. In precompetent larvae mortality did not differ among larvae reared at water temperature from 14.5^{o}C to 29^{o}C. However, larvae reared at a water temperature of 31^{o}C (mean % mortality: 4 ± 0.6%) had an approximate, 3 fold increase in mortality above that observed from larvae reared between 18^{o}C to 26^{o}C (1.2 ± 0.3%). Survival of *O. edulis* larvae reared over a temperature range from 10^{o}C to 32.5^{o}C followed a similar pattern of decreased survival at warmer temperatures (>30^{o}C) (Davis and Calabrese, 1969). *O. angasi* larvae reared at 31^{o}C became competent and underwent metamorphosis, with or without the use of Epi-B. However mortalities increased 4 fold when compared with precompetent larvae reared at 31^{o}C (metamorphosing 13.7 ± 2.8% compared with precompetent 4 ± 0.6%) and increased 4.4 fold compared with metamorphosing larvae reared between 20.5 to 29^{o}C (average 3.1 ± 1.1%). Increased larval growth rates with increasing larval rearing temperature is likely related to a concomitant increase in larval feeding rates to

meet the elevated metabolic demands of faster growth (Rico-Villa et al., 2009). Residual algae counts of the culture water from *O. angasi* larvae reared at 31^{o}C found algal cell concentrations in excess of 10^5 cells mL^{-1} and it is considered unlikely that food availability was a limiting factor for growth and survival in this study. The difference in mortalities observed between precompetent and metamorphosing larvae, reared at the warmest temperature (ie. 31^{o}C), may be a consequence of the metabolic demands for faster growth at higher temperatures exceeding the rate at which energy can be stored given adequate food supply (Gallager et al., 1986). This may increase mortality rates as larvae become reliant on endogenous energy reserves to metamorphose (Holland and Spencer, 1973; Videla et al., 1998). Though no enzyme activity was analysed in this experiment, the differences observed in growth and mortalities with varying temperature, may reflect optimal enzymatic catalytic efficiency within a defined temperature range, outside of which growth may decrease and/or mortalities increase (Lockwood and Somero, 2012). It would be useful to examine the long term (>72h) effects of growing *O. angasi* larvae at warmer water temperatures, because although increased rates of larval metamorphosis of marine invertebrates can occur at warmer temperatures, long term survival of the spat produced has been poor (Nozawa and Harrison, 2007). Although growth was recorded for larvae reared at 18^{o}C or below, no eyed larvae were observed within the time frame of this experiment. Similarly, no spat were obtained from *O. edulis* larvae reared at temperatures <17.5^{o}C (Davis and Calabrese, 1969). Though slower rates of growth and development of *O. angasi* larvae were observed at cooler rearing temperatures (20.5-23^{o}C) compared to larvae reared at warmer temperatures, continued cultivation of larvae at these cooler temperature may result in more individuals successfully metamorphosing (Davis and Calabrese, 1969).

Change of rearing temperature can induce larval metamorphosis in marine invertebrates. However, larval response may depend on the magnitude of temperature alteration, duration of exposure, the absolute temperature elevated to or the direction of the temperature change (Lutz et al., 1970; Boettcher, 2005; O'Connor et al., 2008). Though good growth, survival and metamorphosis were obtained when larvae were reared at 29^{o}C, short term temperature change from 26^{o}C to 29^{o}C decreased larval survival and the number of spat produced. The two different *O. angasi* larval cohorts did not vary in survival or metamorphic response to temperature change, which is encouraging given the large geographic range over which *O. angasi* occurs within Australia (>3000 km) and the potential genetic and environmental variation that could occur over that range. However, care is still required, as the greatest

practical geographic separation achievable in this study (700 km) does not cover the full geographic range of the species and may not reflect overall potential genetic diversity. Additionally, clinal variation in allozymes have been shown to effect adult, and potentially larval survival (Koehn et al., 1976). Accordingly, southern populations of *O. angasi's* larvae may exhibit lower temperature tolerances than larvae from northern populations.

Short term reduction in larval rearing temperature, within the ranges tested, resulted in good larval survival and an increased percentage of spat produced. In this study it is difficult to determine if it is the magnitude of temperature change, the absolute temperature altered to, or duration of exposure that effected metamorphosis of *O. angasi* larvae. Hickman and O'Meley (1988b) suggested that raising the larval rearing temperature from 21^{o}C to 22-23^{o}C led to an increase in metamorphic rate of *O. angasi* larvae. Similarly, *O. edulis* larvae reared between 20^{o}C to 22.5^{o}C resulted in a higher percentage of spat produced compared with larvae reared at higher temperatures (Davis and Calabrese, 1969). It may be that the optimal temperature for competent *O. angasi* larvae to undergo metamorphosis is approximately 23^{o}C. Altering *O. angasi* larval rearing temperature to optimal (ca. 23^{o}C) at competency, regardless of the direction of temperature change, may have a similar result on larval metamorphosis. Further experimentation is required to determine which component of temperature manipulation (e.g. magnitude, absolute or duration) is causal. Though low numbers of spat were produced in this experiment, greater metamorphic success may be achieved when more larvae have attained competency before treatment with Epi-B and temperature manipulation. But more importantly the short term, 1.5^{o}C elevation in larval rearing temperature, equivalent to the normal duration of epinephrine treatment for hatchery produced *O. angasi* larvae (O'Connor et al., 2009), resulted in greater mortalities and less spat produced. This emphasises the necessity to examine the effects temperature change during metamorphosis for hatchery production for different species of bivalve larvae, particularly when any process or treatment is used that may expose larvae to temperature alteration.

5.5 Conclusion

From a hatchery perspective knowing the optimal larval rearing conditions to maximise growth, survival, and development to spat is fundamental to success. Additionally, knowledge of tolerance to changes in physiochemical factors such as temperature and salinity and the consequences of altering these parameters on larval growth, development

and survival are equally important. Our study indicates that *O. angasi* larvae can be reared over a broad range of temperatures but a more defined salinity range. However, particular care must be taken during metamorphosis in maintaining a stable larval rearing environment without prior determination the effects on the species of larvae being reared. The effect of decreasing larval rearing temperature during settlement and metamorphosis on long-term larval survival needs further examination before adopting this process as part of routine hatchery rearing.

The interactive effects of temperature and salinity are well documented (Dove and O'Connor, 2007) and the effect on growth and survival when both stressors are combined can be greater than the effect of the two single stressor added (Heilmayera et la., 2008). Temperature and salinity were assessed separately in these experiments and further work is necessary to determine the interactive effect of both these environmental parameters on *O. angasi* larval growth, survival and development.

The parameters used to rear hatchery seed of this species would ultimately rely on the most cost effective production method for that location. Greatest growth rates and percentage of *O. angasi* spat produced were achieved at a larval rearing temperature between 26^{o}C to 29^{o}C; however the cost of maintaining higher rearing temperatures in colder climates may be prohibitive. Larval growth and spat were still obtained from rearing temperatures between 20^{o}C to 26^{o}C and rearing at these lower temperatures may conducive for cultivation of different larval diets (see O'Connor et al., 2009 for a review of larval diets for this species).

Chapter 6. General discussion

Once a prominent member of coastal subtidal communities around Australia's southeastern coastline, where it formed the basis of a significant fishery, *O. angasi* has for over a half a century been almost a forgotten species ecologically and economically (Alleway and Connell, 2015). Today, interest in restoring *O. angasi* populations (Gillies, 2015) and in commercial production has highlighted the paucity of information regarding this species. This thesis contributes to our knowledge of the biology and ecology of *O. angasi*, particularly in NSW, where farming interest has been the greatest.

Whether the impetus for research is population restoration or farming, attempts to achieve either outcome are by necessity underpinned by hatchery production, which has been limited and frequently unreliable. Two areas of improvement were identified; the need for increased understanding of reproductive activity for larval production and the systematic assessment of larval rearing conditions with particular reference to settlement and metamorphosis. This thesis addresses these knowledge shortfalls and provides industry with fundamental information to improve hatchery production and spat quality. Further, the use metamorphic inducers for assessment of larval rearing environment, assists other hatchery operators to improve and refine larval rearing for *O. angasi*. Increased knowledge regarding reproductive activity of *O. angasi* in Australia will support the move toward much needed breeding program for this species. Work in the thesis has increased our knowledge of the thermal limits for *O. angasi* reproductive activity and has further implications for brood-stock conditioning. More generally, this work adds to the broader understanding of the reproductive activity among the *Ostreinae* and the acquisition of larval competency and metamorphosis in bivalve molluscs. The cumulative improvements in larval rearing technology described in this thesis have reduced the duration of larval rearing from 18-22 (Heasman and Lyall, 2000) days to 10-15 days and increased the percentage of larvae that successfully metamorphose from an historical average of approximately 57% for the four most successful rearing attempts (Heasman and Lyall, 2000) to an overall average of 68% for the nine most recent larval rearing attempts undertaken with the benefit of knowledge obtained during this study.

6.1 *Chapter 2: Ostrea angasi* reproductive activity

The reproductive activity of a species usually follows a defined pattern from a resting phase where nutrients are acquired for gametogenesis, gamete development, ripening, spawning and return to the resting phase (Sastry, 1975). Reproductive activity is thought to be a genetic response to environmental conditions experienced (Giese and Pearse, 1974) and reproduction within a population may be synchronous, with all individual spawning simultaneously or asynchronous, with proportion of the population spawning at any given time during the breeding season (Sastry, 1979). The reproductive activity observed in this study indicates that *O. angasi* is an asynchronous serial spawner and gametogenesis can occur year round in NSW waters. This contradicts previous perceptions of defined seasonal patterns derived from observations from the southern states (O'Sullivan, 1980: Hicks and O'Meley, 1988). It was thought that genetic differences between subpopulations could be a contributing factor to these differences in reproductive activity; however the low level of genetic divergence among Australian *O. angasi* populations does not support this hypothesis (Hurwood et al., 2005).

Continual spawning has been reported for other Ostrieds in the northern extent of their distribution and these differences in reproductive activity were thought to be the result of latitudinal separation (Jeffs, 1998). This may be the case for Victoria and Tasmanian *O. angasi* populations, however South Australian *O. angasi* populations are within the same latitudes as those examined in this study and water temperatures observed in this study are comparable with that previously reported in South Australian waters (Grove-Jones, 1986). Additionally, within the most southern and northern population's sampled, *O. angasi* were found brooding for nine and eight months respectively. Determining the contributing factor or factors to the differences in reproductive activity observed between NSW and South Australian populations of *O. angasi* may give greater insight into the factors controlling the temporal and spatial patterns of reproduction of this species.

From the outset, this study hypothesised defined seasonal peaks in reproductive activity, with a more confined breeding season in southern estuaries and a more protracted breeding season in the most northern estuary sampled. According to the literature (O'Sullivan, 1980), the temperature differential between the estuaries sampled should have indicated the threshold temperature that would synchronise spawning events in the field and that could be applied in

the hatchery. This is an area of research that could form the basis of further targeted laboratory studies.

Though significant associations were found between changes in gametogenic phase and temperature and salinity there was no consistent association across the four estuaries. Sex ratios in oysters are thought to be determined by combination genetic and environmental factors. Important environmental determinants effecting sex ratios in oyster include temperature, food, and day length (Yusa, 2005). *Ostrea edulis* were found to be predominantly female at the start of the breeding season (Joyce et al., 2013). The histological examination, as part of the reproductive assessment of *O. angasi*, also sought to take advantage of the temperature differential between estuaries, observe differences in sex ratios of *O. angasi* and potential changes at the start of the spawning season and relate this to the temperature variations. The asynchronous spawning of *O. angasi* in NSW combined with the ability to rapidly change sex after spawning (O'Sullivan, 1980) may have masked any association of number of individuals in a particular gametogenic phase with the environmental variables measured. The fate of hermaphrodites among *O. angasi* populations could not be determined and whether they were transitional between gametogenic phases or if they contributed directly to spawning events remains unknown. Manipulation of water temperature and salinity under controlled conditions in the laboratory is the next step in elucidating the effect of these two environmental parameters on *O. angasi's* reproductive activity.

6.2 *Chapter 3:* Induced larval metamorphosis

The final measure of larval rearing success is the transition through metamorphosis, which can be difficult to assess. Larvae can delay metamorphosis even though they have achieved competency and a suitable settlement substrate is present (Coon et al., 1990). Without the use of a metamorphic inducer, assessment of larval rearing practices and their effect on rate of larval development to competency is hampered. The development of techniques to induce metamorphosis in competent larvae of any bivalve species offers an important tool for larval research and further expands our understanding of larval metamorphosis.

Not all competent bivalve larvae respond to metamorphosis inducing substances in the same way (Pawlik, 1990; Garcia-Lavandeira et al., 2005): a chemical that induces metamorphosis in larvae of one bivalve species may have no effect or inhibit metamorphosis in other species. Where a common inducer exists, the concentration and duration of exposure to a metamorphic inducing chemical required to elicit a response is often species-specific (Garcia-Lavandeira et al., 2005). This is evident when comparing the response from larvae of two closely related oyster species, *O. edulis* and *O. angasi* (Ketchington et al, 2002; Heasman et al., 2004), to the same metamorphic inducers. GABA induces metamorphosis in *O. edulis* larvae (Garcia-lavandeira et al., 2005) but does not in *O. angasi* larvae (this thesis). Prolonged treatment of *O. angasi* larvae for 24h with epinephrine bitartrate at 10^{-3} M and epinephrine at 10^{-4} M inhibited larval metamorphosis without increased mortality, however long term exposure to concentrations below 10^{-5} for either chemical had no effect on metamorphosis. In contrast *O. edulis* larvae treated with 100 µM epinephrine alone (i.e. no settlement substrate), for 45min to 48h did not affect the number of spat produced (Shpigel et al., 1989) but when *O. edulis* larvae were exposed 10^{-5} M epinephrine for 48h, (24h data not presented) the number of spat produced significantly increased (Garcia-Lavandeira et al,. 2005). Pharmacologically, different receptors types have been found to be involved in the metamorphic pathway in different bivalve larvae (Coon and Bonar, 1987; Wang et al, 2007) and how competency is attained and the mechanisms by which metamorphic inducers work is poorly understood (Garcia-Lavandiera et al., 2005). The divergence is receptor site type is puzzling, however further work in this area may help with determining the mechanisms underlying the development of larval competency.

The non-lethal inhibition of metamorphosis of *O. angasi* larvae may be useful in examination of the biochemical and physical events surrounding this transitional phase in relation to the reaction of other Ostried larvae to the same metamorphic inducing chemical. Exposure of *O. edulis* (Shpigel et al., 1998; Mesias-Gansbiller et al., 2013) and *C. gigas* (Bonar et al., 1990) to the same metamorphic concentrations of epinephrine for 24 to 48h significantly increased larval metamorphosis however metamorphosis of *O. angasi* larvae is inhibited. The mode by which long term exposure to a metamorphic inducer inhibits metamorphosis of *O. angasi* larvae warrants investigation.

6.3 *Chapter 4:* Larval nutrition

The use of epinephrine bitartrate treatment of larvae provided a new tool in the assessment of dietary impacts on survival and the rate of development to competency of *O. angasi* larvae. Few researchers have used induced metamorphosis to examine the effect of larval diet on metamorphic success. As algal production can account for >50% of the running cost of a bivalve hatchery the most efficient use of this commodity is essential. To achieve this end the best single, binary and ternary diet and the benefits of feeding a more complex diet need to be assessed. The production of additional algal species need not be required if one algal species will suffice. If several algal species, fed as single diets gives the same outcome of larval survival and development the hatchery operator can focus on the easiest and most economical algal species to produce and assess the need for additional algal species production.

Accordingly, eight of the most commonly produced algal species were assessed in a series single, binary and ternary diets for survival and development of *O. angasi* larvae. As expected diet had an effect on the rate at which larvae attained competency. Surprisingly, larvae grew as well on the best single species diets as they did on all but the best ternary diet, but those fed the more complex diet attained competency earlier. This implies the use of larval growth rates alone is not the best indicator of dietary suitability for bivalve larvae. Assessment of the dietary requirements for the early brooding stage of larval development, undertaken here, has implications for hatchery production of *Ostreinae* larvae that are produced in conditioning systems. The dietary requirements were similar in both stages of larval development assessed, indicating the diet that gives good performance outside the branchial chamber is most likely to support good performance for brooded larvae.

6.4 *Chapter5:* Effects of water temperature and salinity on larval development

To further refine larval rearing techniques and optimise settlement percentages, the influence of salinity and temperature on development of *O. angasi* larvae was assessed using epinephrine induced metamorphosis. Choice of rearing temperature for any bivalve larvae in a hatchery usually takes in consideration the cost associated with maintaining that temperature at a given location. As is common elsewhere, all hatcheries in NSW are located on an estuarine system, as are a lot of hatcheries elsewhere and the influx of freshwater can lower salinities considerably. Accordingly, the tolerance limits of *O. angasi* larvae for

different rearing temperatures and salinities and their effect on larval survival and metamorphosis needs to be determined.

Knowing the specific tolerance limits of the larvae being produced, and the ramifications of attempting larval rearing outside these parameters, allows planning of seasonal hatchery production to give the best economic outcomes. Indeed, the location of a hatchery may be constrained by the environmental tolerances of the species to be produced. Our study indicates that *O. angasi* larvae can be reared over a broad range of temperatures but a more defined salinity range and hence may restrict larval culture of *O. angasi* depending on the hatchery location and water storage facilities.

Knowledge of tolerance to changes in physiochemical factors such as temperature and the consequences of altering these parameters on larval growth, development and survival are equally important. Particular care must be taken during metamorphosis in maintaining a stable larval rearing environment without prior determination of the effects on larvae of the species reared. Rapid, short term, small fluctuations in water temperature of a few degrees can easily occur in a hatchery. The detrimental effects of a minor increase in larval rearing temperature from a larval rearing of 26^{o}C were evident during epinephrine treatment with increased mortality and decreased survival. However, a short term, minor decrease in temperature, of the same magnitude that was previously detrimental, had a beneficial effect of increased larval metamorphosis. Investigation is needed to determine if it was the magnitude of temperature change, the absolute temperature achieved, or the duration of exposure that effected metamorphosis of *O. angasi* larvae. Further to this, it would be interesting to monitor the effect on induce metamorphosis and temperature manipulation on other bivalve larvae and observe whether a similar response is elicited.

6.5 Conclusion

Given the lack of information available on the reproductive ecology of *O. angasi* in general and in particular NSW this examination of *O. angasi* reproductive activity was warranted. The work in this thesis has redefined the limits of water temperature on the reproductive activity of this species. The redefined temperature limits have implications for the hatchery conditioning of brood-stock and future attempts to manipulate spawning of *O. angasi*.

Similarly the amount of information relating to larval rearing environment on the growth and development of *O. angasi* larvae has been increased. Adopting this approach of assessment of larval rearing environment on development measured by induced metamorphosis is likely to assist in the rearing of other bivalve species.

The development of techniques to induce metamorphosis in competent *O. angasi* larvae has provided an increased supply of single seed to industry, developed an important tool for further larval research with this species and added further information to the broader understanding of larval metamorphosis.

6.6 Future research

While the information presented in this thesis has improved hatchery outcomes and provided useful research tools, it has also identified a number of areas for future research.

To enhance brood-stock management, there is clearly an opportunity to increase our knowledge of the environmental factors influencing gametogenesis and spawning of *O. angasi*.

- In particular, determining temperature limits for gametogenic activity and if temperature manipulation can synchronise spawning events. Additionally the effects of lowered salinities need also be assessed for potential to synchronise spawning (O'Connor et al., 2008) and effects on brooded larvae (Segura et al., 2015).

- Given the importance on broodstock diet on larval growth and survival (Helm et al., 1973; Millican and Helm 1994; Bertsson et al., 1997) examination hatchery diets for conditioning of *O. angasi* and the effect on larval growth, development and survival is warranted in the future.

- Assessment of hatchery conditioning protocols that may influence gender type, beginning with temperature manipulation and different diet regimes (Gonzalez-Araya et al., 2013) would assist with hatchery and breeding line production for all *Ostreinae*. In conjunction with assessment of the hatchery conditioning protocols is the development of successful *in vitro* fertilisation techniques to economically produce

pair-mated breeding lines is warranted. The use of hormonal release of oocytes has not been assessed for *O. angasi* and should be included in future research plans (Wang and Croll, 2003).

- In conjunction with temperature manipulation and controlled diet the use of hormonal application may be effective in controlling sex determination (Nagasawa et al., 2015). The fate of the hermaphrodite phase of *O. angasi* remains unknown; however, if this is a transitional phase between sexes, examination of the hormones controlling this change may shed light on sex determination of *O. angasi.*

- Recent laboratory experiments with *O. edulis* (Joyce et al., 2013), though not completely successful in the desired outcomes, still provide important information that may be useful for conditioning trials with *O. angasi*. This paper offers practical application of different temperature regimes and how reproductive activity, temperature thresholds, sex ratios, and synchrony of spawning for another *Ostreinae*, *O. edulis*. *Ostrea edulis* were found to be predominantly female at the start of the breeding season (Joyce et al., 2013). This information provides as an excellent starting point for hatchery conditioning of *O. angasi*, determination of the lower limit of water temperature that halts reproductive activity and how this affects the sex ratios of this species.

- With respect to larval rearing, the information presented here enables the manipulation of the larval rearing environment to maintain larval growth but slow the onset of competency. How larval competency is attained and the mechanisms by which metamorphic inducers work is poorly understood (Garcia-lavandiera et al., 2005). Onset of competency is thought to be regulated by the development of the appropriate receptor sites (Bonar et al., 1978; Croll et al., 1997; Croll and Dickson, 2004). The use of histochemical staining (Wang et al., 2006) may elucidate where and when the receptor sites develop in relation to induced metamorphosis and ascertain if development of the receptors site are the final physiological change involved in the onset of competency. This knowledge would assist in our

understanding of the metamorphic pathway and the physiological mechanism underlying larval metamorphosis.

- Further assessment of synchronicity of larval development is needed. In particular: do larvae reared under conditions that achieve the onset of competency earlier than their counterparts metamorphose in greater number in a shorter duration?

The development of these techniques and concepts would not only assist in the hatchery production of *O. angasi* but the larval culture of many other bivalves.

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